Characterization of L-serine deaminases, SdaA (PA2448) and SdaB (PA5379), and their potential role in *Pseudomonas aeruginosa* pathogenesis

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38 **ABSTRACT**

39 Regardless of the site of infectivity, all pathogens require high energetic
40 influxes. This energy is required to counterattack the host immune system and in
41 the absence the bacterial infections are easily cleared by the immune system.
42 This study is an investigation into one highly bioenergetic pathway in
43 *Pseudomonas aeruginosa* involving the amino acid L-serine and the enzyme L-
44 serine deaminase (L-SD). *P. aeruginosa* is an opportunistic pathogen causing
45 infections in patients with compromised immune systems as well as patients with
46 cystic fibrosis. L-SD has been linked directly to the pathogenicity of several
47 organisms including but not limited to *Campylobacter jejuni*, *Mycobacterium*
48 *bovis*, *Streptococcus pyogenes*, and *Yersinia pestis*. We hypothesized that *P.*
49 *aeruginosa* L-SD is likely to be critical for its virulence. The genome sequence
50 analysis revealed the presence of two L-SD homologs encoded by *sdaA* and
51 *sdaB*. We analyzed the ability of *P. aeruginosa* to utilize serine and the role of
52 SdaA and SdaB in serine deamination by comparing mutant strains of *sdaA*
53 (PAO*sdaA*) and *sdaB* (PAO*sdaB*) with their isogenic parent *P. aeruginosa* PAO1.
54 We demonstrate that *P. aeruginosa* is unable to use serine as a sole carbon
55 source. However, serine utilization is enhanced in the presence of glycine. Both
56 SdaA and SdaB contribute to L-serine deamination, 34 % and 66 %, respectively.
57 Glycine was also shown to increase the L-SD activity especially from SdaB.
58 Glycine-dependent induction requires the inducer serine. The L-SD activity from
59 both SdaA and SdaB is inhibited by the amino acid L-leucine. These results
60 suggest that *P. aeruginosa* L-SD is quite different from the characterized *E. coli* L-
61 SD that is glycine-independent but leucine-dependent for activation. Growth

62 mutants able to use serine as sole carbon source were isolated. In addition,
63 suicide vectors were constructed which allow for selective mutation of the *sdaA*
64 and *sdaB* genes on any *P. aeruginosa* strain of interest. Future studies with a
65 double mutant will reveal the importance of these genes for pathogenicity.
66

67 INTRODUCTION

68 Amino acids can be used as a carbon source. Most of the amino acids
 69 are converted to pyruvate that enters the Tricarboxylic Acid Cycle (TCA). In the
 70 absence of glucose, microorganisms preferentially utilize L-serine as a carbon
 71 source (Zinser & Kolter, 2000; Zinser & Kolter, 2004; Prüß, 1994). L-serine is
 72 catabolized to pyruvate after a single enzymatic reaction catalyzed by L-serine
 73 deaminase (L-SD) (Prüß, 1994). The pyruvate is converted to acetyl CoA, which is
 74 oxidized via the TCA cycle to carbon dioxide and free energy (Figure 1) L-serine
 75 also serves as an anabolic substrate for vital functions within the cell. It serves as
 76 a monomer for proteins, plays a significant role in phospholipid biosynthesis and
 77 acts as a substrate for the enzymatic synthesis of glycine, cysteine, and
 78 tryptophan (Stauffer, 1996). Glycine, in turn, is a precursor for purines and heme
 79 groups involved in essential nucleic acid formation and redox reactions,
 80 respectively (Stauffer, 1996). Thus, L-serine has a central role in bacterial
 81 metabolism. Fifteen % of the carbon assimilation entering *Escherichia coli*
 82 metabolism from extracellular glucose stores is at one point or another
 83 incorporated into a molecule of serine (Pizer & Potochny, 1964).

84 Despite L-serine's known central role in bacterial metabolism, most
 85 organisms do not utilize L-serine as the sole carbon source (McFall, 1996; Mendz
 86 & Hazell, 1995; Vining & Magasanik, 1981). When in complex media, however, L-
 87 serine is the first amino acid catabolized (Prüß, 1994). Specifically, serine
 88 degradation is greatly enhanced in the presence of small quantities of other
 89 carbon sources such as glucose, glycine, leucine, isoleucine, or threonine

90 (Ogawa *et al.*, 1997). Under these conditions there are two reported phases of
 91 serine utilization during the cell growth cycle (Mendz & Hazell, 1995; Novak, 2000;
 92 Velayudhan & Kelly, 2002). In the first phase most of the serine is metabolized to
 93 pyruvate with the subsequent energy and intermediates contributing to the
 94 growth of the bacterium (Prüß, 1994; Netzer *et al.*, 2004). In the second phase
 95 serine is still consumed, but no further growth is observed (Prüß, 1994; Netzer *et al.*, 2004). The ability to utilize serine in this second phase of growth, allows for
 96 preferential survival by these microorganisms (Zinser & Kolter, 2000).
 97

98 L-serine degradation is preferred over that of glucose in *E. coli* (McFall,
 99 1996). Biochemically, serine degradation is less complex, involving fewer steps
 100 than glucose catabolism. Glucose is broken down via glycolysis (Figure 2) into
 101 two molecules of pyruvate, two adenosine triphosphates (ATP), and two
 102 nicotinamide dinucleotide (NADH) molecules by substrate level phosphorylation
 103 of adenosine diphosphate (ADP). This metabolic process uses 10 enzymes, each
 104 of which is produced by the transcription of their corresponding genes and
 105 translation of the corresponding protein (Stover *et al.*, 2000). The net outcome of
 106 glycolysis is bio energetically favorable. However, in comparison to serine
 107 catabolism, the breakdown of serine is more energetically favorable (Figure 2).
 108 Serine utilization by the organism requires transcription of one gene and
 109 translation of one protein. As compared to glycolysis, serine catabolism bypasses
 110 ten reactions which yield 38 ATP molecules post oxidative phosphorylation, for
 111 one reaction that directly feeds into the Krebs cycle leading to the production of
 112 15 ATPs per pyruvate molecule (Figure 2). There is a 15:1 ATP to enzyme ratio for
 113 L-serine catabolism as compared to a 3.8:1 in glycolysis. This 4 fold increase in

114 bioenergetic efficiency could explain the observed contribution of L-serine
115 catabolism to bacterial pathogenicity (Velayudhan *et al.*, 2004; Chen *et al.*,
116 2003).

117 The enzyme that catalyzes the conversion of L-serine to pyruvate and
118 ammonia is L-Serine Deaminase (Pardee & Prestidge, 1955; Simmonds & Miller,
119 1957; Pardee & Prestidge, 1955). *E. coli* harbors two L-SDs, LSD2 and LSD3
120 encoded by the genes *sdaB*, and *tdcG*. *E. coli* also harbors a third L-serine
121 delaminating enzyme known as L-serine ammonia lyase encoded by *sdaA*, with
122 affinity for both amino acids, threonine and serine (Keseler *et al.*, 2005). In *E. coli*
123 L-SD is translated in an inactive form and activated post translationally via
124 synthesis of a 4Fe4S cluster by the gene products of the *isc* operon (Cicchillo *et*
125 *al.*, 2004). Prokaryotic L-SD differs in its catalytic mechanism from known
126 eukaryotic L-SDs. While eukaryotic L-SD carries out the deamination step via the
127 amino-transferring coenzyme pyridoxal phosphate (Ogawa *et al.*, 1989), the
128 prokaryotic homolog uses an iron sulfur center for catalysis (4Fe-4S) (Cicchillo *et*
129 *al.*, 2004). In *E. coli* the regulation of genes encoding L-SD are under control of
130 the **L**eucine **R**esponsive Regulatory **P**rotein (Lrp) (Newman *et al.*, 1985).

131 In order for a pathogen to successfully colonize its host it must acquire
132 energy from the host. This is most efficiently achieved via secretion of proteases
133 to break down neighboring host proteins to smaller peptides and or individual
134 amino acids. Given the selective advantage conferred on organisms with high L-
135 SD activity *in vitro*, the ability to utilize L-serine released by proteolytic cleavage
136 *in vivo* should confer a selective advantage to these pathogenic organisms as

137 well. Indeed, L-SD has been implicated in the pathogenicity of several organisms,
138 including *Campylobacter jejuni*, *Mycobacterium bovis*, and *Streptococcus*
139 *pyogenes*. In *C. jejuni*, isogenic L-SD mutants failed to colonize the avian gut,
140 while all of the wild type strains inoculated into newborn chickens were able to
141 colonize the cecum (Velayudhan *et al.*, 2004). Thus it appears that L-serine
142 degradation is critical for *C. jejuni* pathogenicity.

143 Any invading organism must break down host metabolites and convert
144 them to free energy, which it can use to sustain its growth in the host. Without the
145 ability to harness energy from these catabolites, such pathogens cannot persist
146 against the massive attacks by the host immune system. The inability to degrade
147 L-serine has been linked to the failure of the live-attenuated vaccine for *M.*
148 *tuberculosis* to persist in its host (Chen *et al.*, 2003). The Bacille Calmette-Guerin
149 (BCG) vaccine, administered worldwide except in the United States, was shown
150 to have an intact gene encoding L-SD, however expression was undetectable
151 (Chen *et al.*, 2003). Lack of L-SD expression led to the accumulation of serine in
152 the cell, which was then shown to inhibit glutamine synthase activity (Chen *et al.*,
153 2003). Thus it has been proposed that the low L-SD expression resulted in the
154 failure of the live vaccine to establish a persistent infection (Chen *et al.*, 2003).

155 Given the selective pressure of host defenses, many pathogenic
156 organisms have devised virulence factors that have allowed them to persist
157 under attack. The production of these virulence factors is often excessive, such
158 as the excess polysaccharide coating produced by many organisms to resist
159 phagocytosis, complement, and opsonization. The production of virulence

160 factors requires energy, and thus some organisms regulate virulence factor gene
 161 expression with metabolic gene expression. For example, *Streptococcus*
 162 *pyogenes* produces the Rgg protein, which serves as a transcriptional regulator
 163 that switches virulence genes on and off as the organism prepares to invade its
 164 host (Chaussee *et al.*, 2003). Besides the expression of virulence factors, it also
 165 regulates the expression of L-SD encoding genes (Chaussee *et al.*, 2003). Thus, a
 166 virulence factor gene regulator manipulates the expression of a metabolic gene
 167 when the bacterium is preparing to infect. This finding adds credence to the
 168 hypothesized contribution of L-SD expression to bacterial pathogenicity.

169 The role of L-SD in the pathogenicity of *P. aeruginosa* has not been
 170 characterized. This Gram negative facultative aerobe is ubiquitous in nature and
 171 is opportunistically pathogenic to burn patients, immunocompromised
 172 individuals, and people suffering from cystic fibrosis (Lyczak *et al.*, 2002). *P.*
 173 *aeruginosa*'s mode of growth protects the organism from antibiotics, as well as
 174 antibodies, complement, and opsonization (Govan & Harris, 1986). Upon
 175 selection by host's defenses, the bacterial population switches from a non-
 176 mucoid to a mucoid form (Mathee *et al.*, 1999). This mucoid form of the
 177 bacterium is virtually untreatable with traditional antibiotics, making it necessary
 178 to seek alternate modes of attack (Hill *et al.*, 2005). Since all pathogenic
 179 organisms need to obtain energy from host metabolites, if you cut off this energy
 180 source, the bacteria are left without the ability to produce the virulence factors
 181 contributing to their defense against the host. Thus, energy-producing metabolic
 182 pathways may provide a new drug target for treatment of *P. aeruginosa*
 183 infections.

184 Little is known about L-serine degradation in *P. aeruginosa*. However,
 185 genome sequence analysis reveals the presence of two L-SDs encoded by the
 186 genes *sdaA* (PA2448) and *sdaB* (PA5379) (Stover *et al.*, 2000). The *P. aeruginosa*
 187 L-SD proteins share the conserved cysteine residues involved in the stabilization of
 188 a catalytic iron sulfur center (Flint & Allen, 1996). This data indicates that these
 189 enzymes, like their prokaryotic homologs, most likely exhibit catalysis via an iron
 190 sulfur cluster. Given the similarity of the *P. aeruginosa* homologs to the L-SD
 191 proteins implicated in pathogenicity, we postulate that *P. aeruginosa* L-SD plays
 192 a similar role in the physiology of *P. aeruginosa*.

193 The pathogenicity of *P. aeruginosa* is of interest not only in CF patients
 194 who ultimately succumb to the infection, but also in burn patients, and in
 195 patients acquiring *Pseudomonas* nosocomial (hospital-acquired) infections.
 196 Heavy emphasis on this organism's resistance to both bacteriostatic and
 197 bactericidal treatment has elucidated a heavy arsenal of antibiotic resistance
 198 mechanisms which make classical treatment with antibiotics ineffective at best.
 199 The failure to treat patients with these infections, fuels the drive to explore new
 200 areas upon which we might effectively hinder or even eliminate the bacterium's
 201 growth *in vivo*. In light of the recent elucidation of the role of L-SD in the
 202 pathogenicity of several organisms (*S. pyogenes*, *M. bovis*, *C. jejuni*), we
 203 hypothesize that the L-SD in *P. aeruginosa* confers virulence. *P. aeruginosa*
 204 harbors two genes that encode L-SD, *sdaA* and *sdaB*. The aims of this study are
 205 to characterize L-serine utilization by *P. aeruginosa*, determine the contribution of
 206 *sdaA* and *sdaB* to the total L-SD activity, and investigate *P. aeruginosa* L-SD
 207 contribution to its virulence

208 MATERIALS AND METHODS

209 **Bacterial and Nematode Strains, Plasmids and Media.** Genotypes and
 210 sources of bacterial and nematode strains, plasmid genotypes and sources used
 211 in this experiment are listed in Table 1. *P. aeruginosa* and *E. coli* strains were
 212 cultured in Luria-Bertani (LB) broth or LB agar at 37 °C unless otherwise stated.
 213 Antibiotics for *E. coli* were added at the following concentrations: ampicillin (Ap),
 214 50 µg/ml, carbenicillin (Cb), 10 µg/ml, gentamycin (Gm), 15 µg/ml, kanamycin
 215 (Km), 50 µg/ml, tetracycline (Tc), 20 µg/ml. For *P. aeruginosa* Cb, 300 µg/ml, Gm,
 216 300 µg/ml, Nm, 150 µg/ml, and Tc, 60 µg/ml were used. Nematodes were
 217 cultured in Nematode Growth Media unless otherwise stated.

218 **Protein Sequence Analysis of *P. aeruginosa* L-SD.** Protein sequence
 219 homology analysis was performed between the two L-SDs encoded by *P.*
 220 *aeruginosa* and several L-SD homologs in bacterial species of interest utilizing the
 221 program Protean, DNA STAR, 2004. These proteins and bacteria consisted of *E.*
 222 *coli* SdaA and SdaB, *Mycobacterium tuberculosis* SdaA, *M. bovis* SdaA,
 223 *Campylobacter jejuni* SdaA, *Streptococcus pyogenes* SdaA, and *Yersinia pestis*
 224 SdaA. Further analysis of SdaA and SdaB was performed via the computer
 225 program Protean, DNA Star, 2004. Hydrophobicity plots for SdaA and SdaB were
 226 performed utilizing the following Colorado State University website:
 227 <http://www.vivo.colostate.edu/molkit/hydropathy/index.htm>

228 **Chromosomal DNA Extraction.** A modification of the Qiagen Genomic
 229 DNA Kit (Qiagen Inc., Valencia, CA) was used to extract *P. aeruginosa*
 230 chromosomal DNA (Qiagen Inc., Valencia, CA). Strains of interest were grown

overnight at 37 °C. One milliliter of this culture was centrifuged and resuspended in Buffer B1 (Bacterial Lysis Buffer: 50 mM Tris-HCl, 50 mM EDTA, 0.5 % Tween-20, 0.5 % Triton X-100, pH 8.0) and 2- µl RnaseA (100 µg/ml). Twenty microliters of lysozyme (100 mg/ml) was added, followed by 45 µl Proteinase K (20 mg/ ml). The suspension was incubated at 37 °C for 30 minutes, then 175 µl of Buffer B2 (Bacterial lysis buffer: 3 M guanidine HCl, 20 % Tween-20) was added. The tubes were inverted several times and incubated at 50 °C for 30 minutes. An equal volume of phenol/chloroform was added, followed by vortexing and centrifugation. A 0.1 volume of 3 M sodium acetate and 2 volumes of ice cold 70 % (v/v) ethanol were added to the decanted supernatant and incubated for 30 minutes at -20 °C. The DNA-containing solution was vacuum dried and resuspended in 50 µl of dH₂O.

Gene Amplification. To obtain a sufficiently high quantity of DNA for cloning, genes of interest were amplified via the polymerase chain reaction (Techne, Albany, NY). To amplify *sdaA*, the primer pairs PAO*sda*AF, PAO*sda*AR and PAO*sda*AH3F, PAO*sda*AH3R were used (Table 1). To amplify *sdaB*, the primer pair PAO*sda*BF and PAO*sda*BR were used (Table 1).

The parameters used to amplify both *sdaA* and *sdaB* from *P. aeruginosa* chromosomal DNA are as follows: initial denaturation of chromosomal DNA occurred at 95 °C. This step was followed by 30 cycles of denaturation for 1 minute at 95 °C, annealing for 1 minute at 63 °C, and extension for 4 minutes at 72 °C. The final extension occurred at 72 °C for 10 minutes followed by a final hold at 4 °C.

254 To amplify the Km resistance cassette *ahcI* from the EZ-Km transposon kit
255 the same PCR parameters were used except for the primer extension time that
256 was set at 1 minute. The primers used were EZ-Km*Stu*IF and EZ-Km*Stu*IR (Table 1).

257 **DNA Isolation.** In order to obtain pure DNA, the fragments of interest were
258 separated via electrophoresis on a 1 % agarose gel made with TBE buffer. DNA
259 was stained with either ethidium bromide or the non-carcinogenic alternative
260 SYBR SAFE (Invitrogen, Carlsbad, CA) and visualized under UV light. DNA
261 fragments were excised from the gel with a scalpel. The gel fragment containing
262 the DNA was weighed out and three volumes of Buffer QG (Solubilization and
263 Binding Buffer) were added to the gel fragment. The mix was incubated at 50 °C
264 until the solution was homogeneous; one gel volume of isopropanol was added
265 and the mixture was vortexed. This solution was applied to the QIAspin column
266 filter (Qiagen Inc., Valencia, CA) and centrifuged for 1 minute at 13,000 rpm. The
267 filtrate was discarded and the column was washed using 750 µl of Buffer PE (100
268 ml Buffer PE, 400 ml ethanol) and centrifuged for one minute. The filtrate was
269 discarded and a second spin performed to remove residual ethanol in the
270 column. Thirty to 50 µl of Buffer EB (Elution Buffer; 10 mM Tris-HCl, pH 8.5) was
271 added. Following a 1-minute incubation period, a final spin eluted the purified
272 DNA of interest.

273 **Plasmid DNA Extraction.** In order to extract plasmids of interest from the
274 cells harboring them, the Qiaprep Spin Miniprep Kit was used (QIAGEN
275 Inc.,Valencia, CA) Strains harboring the plasmids were grown overnight in 10 ml
276 LB with appropriate antibiotic. The cells were harvested by centrifugation at

277 8,000 x g for 6 minutes. The pellet was resuspended in 500 µl Buffer P1
 278 (Resuspension buffer; 50 mM Tris-Cl, pH 8.0, 10 mM EDTA; 100 µg/ml RNaseA)
 279 followed by the addition of 500 µl of Buffer P2 (Lysis Buffer; 200 mM NaOH, 1 %
 280 Sodium Dodecyl Sulfate). The tubes were inverted to gently mix the solution and
 281 incubated at room temperature (RT) for 5 minutes before adding 750 µl of Buffer
 282 N3 (Neutralization Buffer; 3.0 M potassium acetate, pH 5.5) to neutralize the
 283 reaction. Two ml aliquots were transferred to micro centrifuge tubes and then
 284 centrifuged at 13,000 x g. The supernatant containing the DNA was decanted
 285 into a QIA prep spin column filter. The column was centrifuged and washed with
 286 750 µl of Buffer PE (100 ml Buffer PE, 400 ml 100 % ethanol). The DNA was eluted
 287 into a new micro centrifuge tube after incubation for one minute at 25 °C with 50
 288 µl of Buffer EB (Elution Buffer; 10 mM Tris-HCl, pH 8.5) and a final spin down.

289 **Preparation of Competent Cells and Chemical Transformation.** To make
 290 chemically competent *E. coli* DH5a cells for plasmid transformation, DH5a was
 291 cultured in 5 ml of LB broth at 37 °C with shaking. After overnight incubation a
 292 1:100 subculture into 50 ml of LB broth was incubated till an OD₆₀₀ value of 0.5 to
 293 1.0 was reached. The cells were centrifuged at 6000 rpm in a refrigerated micro
 294 centrifuge set to 0 °C. The supernatant was decanted and to the cells were
 295 added 25 ml of ice-cold 0.1 M CaCl₂. The suspension was mixed via tube
 296 inversion. The cells were then chilled on ice for 15 minutes and centrifuged once
 297 more as stated above. The cells were then resuspended in 3.3 ml of sterile, ice-
 298 cold 0.1M CaCl₂-15% glycerol, and left on ice for 4 to 20 hours. Fifty microliter
 299 aliquots were transferred to micro centrifuge tubes and either used immediately
 300 to transform plasmid DNA of interest or stored at -70 °C up to several months.

301 Ten μ l of ligated or vector DNA was added to the competent cells and
 302 incubated on ice for 15 minutes. The cells were then heat shocked for 2-3
 303 minutes at 37 °C and immediately placed on ice for 5 minutes. One ml of LB
 304 broth was added and the culture placed in a shaking incubator at 37 °C for one
 305 hour to allow time for transformant recovery to take place. Post incubation, 100
 306 μ l of this mix was plated on selective media. The remaining cells were spun
 307 down, resuspended in 100 μ l of LB, and also plated on selective media. The cells
 308 were then incubated overnight at 37 °C and observed the following day.

309 **Preparation of Electrocompetent Cells and Electroporation.** In order to
 310 prepare electrocompetent cells 500 μ l of overnight culture was inoculated into
 311 500 ml of LB broth. The cells were grown at 37 °C with shaking at 250 rpm to an
 312 OD₆₀₀ of approximately 0.6. The cells were chilled for 20 minutes; then
 313 centrifuged at 4000 g for 15 minutes at 4 °C. The cell pellet was resuspended
 314 and washed with 500 ml of ice-cold 10 % (v/v) glycerol, centrifuged and
 315 resuspended in 20 ml of the same medium. This suspension was centrifuged and
 316 resuspended in 2 ml ice cold 10 % glycerol. Forty microliter aliquots were
 317 transferred to micro centrifuge tubes for storage at -70 °C for later use.

318 Frozen electrocompetent cells were thawed (40 μ l) and 2 μ l of the DNA of
 319 interest was added. The cells were incubated for 1 minute on ice and transferred
 320 to a 0.2 cm gene pulser cuvette (BIORAD, Hercules, CA), at which point the tube
 321 was placed in the electroporation chamber and electroporated at setting *E. coli*
 322 2 on the BIORAD Micropulser (BIORAD, Hercules, CA). Immediately after pulsing,
 323 1 ml SOC medium (2.0 % tryptone, 0.5 % yeast extract, 10 mM MgCl₂, 10 mM

NaCl, 2.5 mM KCl, 10 mM MgSO₄, 20 mM glucose) was added and the suspension transferred to a new micro centrifuge tube. This culture was incubated for 30 min at 37 °C with shaking. It was spun down and resuspended in 100 µl of LB medium. Fifty microliters of this culture was spread on selective media and incubated overnight at 37 °C.

Plasmid Construction. Plasmid constructs were made which allow for selective inactivation of *sdaA* and *sdaB* within the *P. aeruginosa* chromosome.

Construction of *sdaA::aacCI*, pXSMLA. To force homologous double crossover recombination at the wild type *sdaA* locus in the *P. aeruginosa* chromosome, a suicide vector (capable of chromosomal allelic replacement) carrying an insertionally inactivated $\Delta sdaA:aacI$ cassette was constructed.

The first step in suicide vector formation involved amplification of the *sdaA* gene with primers PAO*sdaA*F and PAO*sdaA*R (Table 1). Taq DNA polymerase leaves 3'-deoxyadenosine residues during the amplification reaction and pCR2.1 has engineered 3' deoxythymidine residues allowing for easy annealing and ligation using topoisomerases to occur (Invitrogen, Carlsbad, CA.). The 2244 bp amplicon was cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) (Figure 3). The reaction involved a 5-minute incubation at 25 °C with 4 µl of PCR-amplified DNA, 1 µl of 10X Reaction Buffer, and 1 µl of the vector pCR2.1. The ligation mix was used to transform TOP10F' cells (Invitrogen, Carlsbad, CA). Upon DNA insertion into pCR2.1's cloning site, the *lacZ* gene was disrupted, allowing selection of recombinants based on blue-white screening. Transformants were plated on LB containing Ap (50 µg/ml) plates that were spread with 40 µL of 5-

347 Bromo-4-chloro-3-indolyl- β -D galactopyranoside (X-gal, 40 mg/ml) and 40 μ l
348 Isopropyl β -D-thiogalactopyranoside (IPTG, 100 mM; only white transformant
349 colonies were selected. Confirmation of the correct clone was carried out via
350 gel electrophoresis of the *Eco*R1 digest on a 1 % agarose gel in TBE Buffer. The
351 cloned pCR2.1 derivative with the *sdaA* gene insert was named pCSMLA (Figure
352 3).

353 This plasmid was derived via sub cloning the 855-bp *Sma*I fragment
354 containing *aacI* encoding gentamycin resistance, from pUCGM (Schweizer,
355 1993) into a unique *Sma*I site on the *sdaA* gene on pCSMLA (Figure 3). The blunt
356 end ligation was carried out via overnight incubation with 10 μ l Ligase Buffer, 2 μ l
357 T4 ligase, and 0.01 μ M of *Sma*I digested *aacI* and 0.090 μ M of the *aacI* cassette,
358 10:1 molar ratio (New England Bio labs Beverly, MA). Electrocompetent DH5a
359 cells were electroporated with 2 μ L of the ligation mix, and transformants
360 selected on LB plates containing Ap.

361 Transformants were grown in LB Broth with Gm (15 μ g/ml). Confirmation of
362 the correct clone was carried out via gel electrophoresis of the *Eco*R1 digest on
363 a 1 % agarose gel in TBE Buffer. The pCSMLA derivative with *sdaA:aacI* was
364 named pCSMLA2 (Figure 3).

365 A 3,117-bp *Hind*III-*Xba*I-Klenow treated fragment containing the
366 *sdaA:aacI* fragment was sub cloned into a *Sma*I site on pEX100T (Figure 3). The
367 transformants were selected on LB agar plates containing Ap (50 μ g/ml).
368 Confirmation of the correct clone was carried out via gel electrophoresis of the
369 *Fsp*I digest on a 1 % agarose gel in TBE Buffer. The pEX100T derivative was named
370 pXSMLA.

371 **Construction of *sdaB::aphI*, pXSMLBK.** To force homologous double
 372 crossover recombination at the wild type *sdaB* loci in the *P. aeruginosa*
 373 chromosome, a suicide vector carrying an insertionally inactivated $\Delta sdaB:aphI$
 374 cassette, pXSMLBK, was constructed (Figure 4). The *sdaB* gene was PCR
 375 amplified from the PAO1 chromosome using the primer pair PAOsdaBF and
 376 PAOsdaBR (Figure 4, Table 1). The amplicon was sub cloned into pCR2.1 forming
 377 pCSMLB (Figure 4).

378 The 2049-bp *EcoR*I DNA bearing *sdaB* from pCSMLB was sub cloned into
 379 *EcoR*I-cut pUC19 forming pUSMLB (Figure 4). The switch from pCR2.1 to pUC19
 380 was carried out because pUC19 lacks the kanamycin resistance marker (*aphI*). A
 381 second *aphI* cassette was later used to insertionally inactivate the *sdaB* gene.

382 A 979-bp fragment containing *aphI* encoding kanamycin resistance was
 383 amplified from the EZ-transposon kit using the primer pair EZ-Km*Stu*I_F and EZ-
 384 Km*Stu*I_R (Figure 4, Table 1). The amplicon was digested with *Stu*I and sub cloned
 385 into a *Stu*I cut pUSMLB forming pUSMLBK (Figure 4). A 3010-bp *EcoR*I-Klenow
 386 fragment containing *sdaB:aphI* from pUSMLBK was ligated to a *Sma*I cut pEX100T
 387 forming pXSMLBK. (Figure 4). Transformants were selected on LB + Km (50 µg/ml)
 388 and the clone confirmed via *Fsp*I digest.

389 **Construction of *sdaB::aacCI*, pXSMLBG .** Another suicide vector, pXSMLBG,
 390 with *sdaB* insertionally inactivated using a gentamycin cassette (*aacI*) was
 391 constructed (Figure 5). A 855-bp *aacI* DNA fragment was sub cloned into the
 392 *Stu*I site of pUSMLB forming **pUSMLBG** (Figure 5). This plasmid was then digested
 393 with *EcoR*I, releasing two fragments of 2686 bp and 2886 bp respectively. The
 394 2886-bp fragment containing the *sdaB:aacI* cassette was ligated to *Sma*I cut

395 pEX100T forming **pXSMLBG** (Figure 5). The presence of the right clone was
396 confirmed via *FspI* digestion.

397 **Construction of Complementing Plasmids.** In order to construct
398 complementing plasmids with *sdaA* and *sdaB*, the stable, broad host range, low
399 copy plasmid, pME6030, was used (Heeb *et al.*, 2000).

400 **pMEsdaA:** A 2250-bp *HindIII* fragment bearing *sdaA* was purified from
401 pCSMLAH3 and ligated to *HindIII*- cut pME6030 (Figure 6). Confirmation of the
402 correct clone was carried out via gel electrophoresis of the *HindIII* digest on a 1
403 % agarose gel in TBE Buffer. The pME6030 derivative with *sdaA* was named
404 pMEsdaA (Figure 6).

405 **pMEsdaB:** A 2049-bp *EcoRI* fragment harboring *sdaB* was transferred from
406 pUSMLB to *EcoRI*-cut pME6030 (Figure 6). Confirmation of the correct clone was
407 carried out via gel electrophoresis of the *PvuII* digest on a 1 % agarose gel in TBE
408 Buffer. The pME6030 derivative with *sdaB* was named pMEsdaB (Figure 6).

409 **Construction of Suicide Vectors for Mutant Construction**

410 **Sub cloning *sdaA* and *sdaB* from the PAO1 Chromosome.** The *sdaA* gene
411 was successfully amplified from the PAO1 chromosome using two different primer
412 pairs. The 2346-bp fragment amplified using PAOsdaAH3F and PAOsdaAH3R was
413 referred to as *sdaAH3* (Figure 7A, Lane 2). A second fragment containing *sdaA*
414 was amplified (2334 bp) with the primer pairs, PAOsdaAF and PAOsdaAR (data
415 not shown). The *sdaB* gene was also successfully amplified (2049 bp) from the
416 PAO1 chromosome using the primers PAOsdaBF and PAOsdaBR (Figure 8, Lane
417 2).

418 All three amplicons (*sdaA*, *sdaAH3*, and *sdaB*) were ligated to vector
 419 pCR2.1 (forming pCSMLA, pCSMLAH3, and pCSMLB) (Figure 3, 4, 5). *EcoR*I
 420 restriction enzyme digest of pCSMLA yielded three confirmatory fragments of
 421 743, 1515, and 3913 bp (data not shown). A *Hind*III restriction enzyme digest of
 422 pCSMLAH3 yielded three confirmatory fragments of 67, 2250, and 3882 bp
 423 (Figure 7A, Lane 3). Finally, *EcoR*I digest of pCSMLB yielded two confirmatory
 424 fragments of 2049 bp and 3913 bp (Figure 8, Lane 3).

425 **Confirmation of Complementary Plasmids.** The 2250-bp *Hind*III fragment
 426 containing *sdaA* from pCSMLAH3 was ligated *Hind*III-cut pME6030 (Figure 7B,
 427 Lane 2,4) (Figure 3). *Hind*III digestion of the clone (pME*sdaA*), confirmed the
 428 presence of two amplicons 2250 and 8021 bp (Figure 7B, Lane 6). The *sdaB*
 429 amplicon was cloned into the vector pCR2.1 yielding pCSMLB (Figure 4). pCSMLB
 430 yielded two fragments of 2049 and 3913 bp when digested with *EcoR*I (Figure 8,
 431 Lane 3). The 2049-bp *EcoR*I DNA was successfully ligated to pME6030-*EcoR*I
 432 (Figure 7B, Lane 3) (Figure 6). pME6030- *sdaB* (pME*sdaB*) transformants were
 433 characterized by Tc^R and screened via an *EcoR*I digest revealing two bands of
 434 2049 bp and 8021 bp (Figure 7B, Lane 7).

435 **Transfer of *sdaB* to a Km^S Plasmid.** The 2049-bp *EcoR*I fragment from
 436 pCSMLB (Figure 4) was successfully ligated to *EcoR*I-cut pUC19. The transformant
 437 phenotype for pUC19-*sdaB* (pUSMLB) was the following: Ap^R, Km^S, and white
 438 colony formation on growth with X-gal. *EcoR*I digest of pUSMLB (Figure 4),
 439 yielded the expected fragments of 2049 bp and 2686 bp (Figure 8, Lane 5).

440 **Insertional Inactivation of *sdaA* and *sdaB* via an *aacCI* Cassette.** The 855-
 441 bp *Sma*I fragment from pUCGm containing the *aacCI* gene encoding
 442 gentamycin resistance, was ligated to *Sma*I-cut pCSMLA (Figure 3) and a *Stu*I
 443 cut pUSMLB (Figure 5) (Figure 8, Lane 7 and 9, Figure 7A, Lane 5). *Hind*III-*Xba*I
 444 double digest of the Gm^R pCSMLA::*aacCI* plasmid (pCSMLA2), yielded two
 445 confirmatory fragments of 3211-bp and 3819-bp (Figure 7A, Lane 6).
 446 pUSMLB::*aacI* (pUSMLBG) yielded two confirmatory fragments of 2686 and 2904
 447 bp when cut with *Eco*RI (Figure 8, Lane 11).

448 **Insertional Inactivation of *sdaB* via an *aphI* Cassette.** The 979-bp *aphI*
 449 gene, encoding kanamycin resistance, was successfully amplified from the EZ
 450 transposon kit using the primer pair EZKm*Stu*IF and EZKm*Stu*IR (Figure 8, Lane 7).
 451 The *aphI* amplicon was restricted with *Stu*I and ligated to *Stu*I cut pUSMLB
 452 yielding transformants with the following phenotype: Ap^R, Km^R, white on X-gal
 453 selection (Figure 8, Lane 9). *Eco*RI digest of the pUSMLB-*aphI* clone (pUSMLBK)
 454 revealed two confirmatory fragments of 2686 bp and 3010 bp (Figure 4) (Figure
 455 8, Lane 10).

456 **Suicide Vectors Bearing *sdaA*::*aacCI*, *sdaB*::*aacCI*, and *sdaB*::*aphI*.**
 457 Fragments containing *sdaA*::*aacCI*, *sdaB*::*aacCI*, and *sdaB*::*aphI* were sub
 458 cloned into the suicide vector pEX100T from pUSMLBK, pUSMLBG, and, pCSMLA2
 459 respectively (Figure 8, Lane 12 and 13, Figure 7A, Lane 7). As expected, a *Hind*III
 460 and *Xba*I double digested pCSMLA2 yielded two linear DNA bands of 3211 and
 461 3819 bp (Figure 7A, Lane 6). *Eco*RI digestion of pUSMLBG, yielded two linear DNA
 462 fragments of 2686 bp and 3010 bp (Figure 8, Lane 13). Similarly, *Eco*RI digested of

463 pUSMLBK yielded two linear DNA bands of 2686 bp and 2904 bp (Figure 8, Lane
464 12).

465 The blunt-ended fragments representing the *sdaA::aacCI*, *sdaB::aacCI*
466 and the *sdaB::aphI* cassettes of 3211 bp, 2904 bp, and 3010 bp, respectively,
467 were ligated to *SmaI*-pEX100T (Figure 8, Lane 12 and 13, Figure 7A, Lane 7).
468 Transformants were Ap^R, Gm^R, and appeared white on X-gal selection. *FspI*
469 digestion of the pEX100T- *sdaA::aacCI* clone (pXSMLA) (Figure 3) yielded two
470 confirmatory linear DNA fragments of 4183 bp and 4874 bp (Figure 7A, Lane 9).
471 *FspI* digest of the pEX100T- *sdaB::aacCI* clone (pXSMLBG) (Figure 5) yielded four
472 confirmatory linear DNA fragments of 363, 1023, 1608, and 2596 bp when
473 subjected to gel electrophoresis (Figure 8, Lane 15). *FspI* digest of the pEX100T-
474 *sdaB::aphI* clone (pXSMLBK) (Figure 4) yielded four confirmatory linear DNA
475 fragments of 363, 1023, 1714, and 2596 bp.

476 **Determination of the Ability of *P. aeruginosa* to Grow on L-Serine.** In order
477 to determine if *P. aeruginosa* could utilize serine as the sole carbon source, all
478 strains of interest (PAO1, PAO*sdaA*, and PAO*sdaB*) were grown overnight in NIV-
479 Glucose (NIVGlu) media broth. Strains PAO1, PAO*sdaA*, and PAO*sdaB* were
480 grown overnight in LB media and sub cultured in NIV glucose media overnight.
481 Cells were resuspended in saline, diluted 10⁻⁶, and plated on differential media
482 which included NIV Glu, NIV SGL (Serine, Glycine, and Leucine), NIV SG (Serine
483 and Glycine), NIV SL (Serine and Leucine), NIV S (Serine), NIV G (Glycine), NIV L
484 (Leucine). As a control the cells were also plated on NIV and NMM media.
485 Colonies were observed for three days and colony size and count recorded
486 three days' post incubation.

487 **Serine Efficient Mutant Strain Isolation and Characterization.** Mutant strains
488 capable of growth on minimal media with L-serine as the sole carbon source
489 were isolated via prolonged growth and continuous restreaking on NIV S media.
490 The purified mutant strains were grown for 36 hours in NIV S media at which point
491 in time the OD₆₀₀ value was recorded. These mutants were then subjected to L-
492 SD assays after 14 hr. growth on NIV S and NIV SGL media.

493 ***P. aeruginosa* Growth Curves in Minimal Media** PAO1, PAOsdA, and
494 PAOsdB were streaked on LB agar plates +/- the addition of antibiotics, and
495 incubated overnight. Single colonies were cultured in NIV Glu overnight. The cells
496 were spun down at 5000 rpm for 6 minutes at 4 °C. The supernatant was
497 decanted and the cells washed twice with sterile dH₂O. The cells were then
498 resuspended in the appropriate minimal media. The cultures were incubated at
499 37 °C with aeration. OD₆₀₀ values were recorded every hour for 16 hours.

500 **L-SD Assay Optimization.** To establish a method of analyzing the amount
501 of L-serine being utilized within a *P. aeruginosa* whole cell, the *E. coli* L-SD assay
502 was modified (Newman & Kapoor, 1980; Isenberg & Newman, 1974). PAO1 was
503 grown in NIV GluG and NIV SGL media for 12 hours. The L-SD assay described for
504 *E. coli* was carried out with the reaction being stopped at 10 minute intervals for
505 100 minutes (Isenberg & Newman, 1974).

506 ***P. aeruginosa* L-SD Assay.** All strains of interest were grown in LB media
507 with appropriate antibiotics overnight at 37 °C. The next day a 1/100th subculture
508 was transferred to NIV media (Serine, Glycine, and Leucine (SGL), Serine and
509 Glycine (SG), Serine (S), Glucose and Glycine (GluG), Glucose and Leucine

(GluL), Glucose (Glu), and LB Media). At 14 hr, the culture was cooled in an ice water suspension for one minute with swirling. The culture was centrifuged and resuspended in KPO₄ buffer (50 mM, pH 7.5). The OD₆₀₀ value of the suspension was adjusted to 0.300 +/- 0.010. Three hundred microliters of the adjusted cell suspension were added to five pre-warmed test tubes (1 hr. at 37 °C) containing 180 µl of 50 mM KPO₄ with or without the addition of 20 µl of 10 % L-serine. Twenty microliters of toluene were added and the test tubes were briefly vortexed and placed in a 37 °C incubator without aeration for 50 minutes. At 50 minutes, 900 µl of dinitrophenylhydrazine (DNPH) solution was added, and the test tubes were incubated at 25 °C for an additional 20 minutes. At twenty minutes the reaction was stopped via addition of 1.9 ml of NaOH (10 % w/w). The OD₅₄₀ values were read on the BIORAD SmartSpec3000 and converted to µg of pyruvate via the pyruvate standard curve described below.

During the assay L-serine is converted to pyruvate by *P. aeruginosa* during the 50 minute incubation at 37 °C. DNPH is allowed to react with the pyruvate in solution. and upon NaOH addition, a colorimetric change is observed. The extent of the change correlates with the amount of pyruvate produced and hence the amount of L-serine deaminated by the *P. aeruginosa* cells.

Pyruvate Standard Curve. Varying concentrations of sodium pyruvate (0, 0.5, 1, 2, 3, 4, and 5 µg/ml) were assayed to create a standard curve by which L-SD assay OD₅₄₀ values could be converted to µg of pyruvate in solution. To do this, 900 µL of DNPH was added to 1 mL of the above stated sodium pyruvate solutions. The mixture was allowed to incubate for 20 minutes, after which 1.9 mL

533 of 10 % NaOH was added to stop the reaction. The OD₅₄₀ values were recorded
534 and a standard curve drawn.

535 The data for the pyruvate standard curve was plotted with pyruvate
536 concentrations on the Y axis and OD₅₄₀ values on the x axis. The slope intercept
537 equation obtained from this graph ($y = 21.117x + 0.1599$) was then used to
538 calculate an extended version of the sodium pyruvate standard curve. This
539 extended version of the pyruvate standard curve was necessary to extrapolate
540 OD₅₄₀ readings for pyruvate concentrations greater than 5 µg/ml. Regression
541 analysis of the linear graph revealed an R² value of .9824. The slope intercept
542 equation obtained from the graph above ($y = 21.117x + 0.1599$) was used to
543 calculate an extended version of the Sodium Pyruvate Standard Curve. This
544 extended standard curve was utilized in subsequent experiments to convert
545 OD₅₄₀ values to µg of pyruvate. The slope intercept equation for this graph is the
546 following: $y = 21.222x + 0.116$.

547

548 RESULTS

549

550 ***P. aeruginosa* Genome Sequence Analysis for the Presence of L-SDs. *P.***

551 *aeruginosa* genome sequence analysis reveals the presence of two open
552 reading frames (ORFs) encoding L-SD homologs, PA2443 and PA5379 (Figure 9).
553 They are located at position 2742353 to 2740977 and 6056877 to 6058253,
554 respectively (Stover *et al.*, 2000). These ORFs are also referred to as *sdaA* and
555 *sdaB*, respectively.

556 The sequence analysis around *sdaA*, revealed the presence of a possible
557 promoter start site between PA2449-PA2447 with a score of 0.92 on a scale in
558 which the maximum is one and a cut off of 0.8 is deemed an accurate
559 prediction of promoter sequence credibility (Reese, 2001). According to this
560 bioinformatic analysis, the minimal promoter region encompasses the following
561 sequence 5' CTGGGATTGACCAGTTGCAGGTGGGTAATTCGAATGGGCGATGCC
562 GCTGT ...3' with the -35 and -10 recognition sequences being 5' TTGACC 3' and
563 5' TCGAAT 3', respectively. This promoter region is found upstream of four genes.
564 The analysis of the intergenic region between *sdaA* and its downstream
565 neighboring gene *gcvT2*, revealed the presence of an inverted repeat followed
566 by a string of uracil (5'ACCGCGCCGCTGCGCCGGCGCTTCTTTTCTTT 3';
567 underline indicates the inverted repeat) denoting Rho-independent
568 transcriptional termination (Roberts, 1969). The *sdaA* gene appears to be the last
569 gene in a large operon in which the promoter region is found four genes
570 upstream and transcriptional termination occurs immediately after *sdaA* (Figure
571 9A).

572 The sequence analysis around *sdaB* revealed the presence of a possible
 573 promoter region (Score = 0.69), 744-bp upstream of *sdaB* (Reese, 2001). Thus, the
 574 predicted *sdaB* promoter region is localized between 6,056,088 bp and 6,056,133
 575 bp (5' CTCCAGGTTGGCGCGCAGTGTCTCGACCGTACCTTCTCGG CGTACTGCT
 576 3') with the -35 and -10 recognition sequences being 5' TTGGCG 3' and 5'
 577 TACCTT 3', respectively. Analysis of the intergenic region between *sdaB* and its
 578 downstream ORF PA5380, revealed the presence of an inverted repeat followed
 579 by a string of uracil at position 6058497 to 6058520 namely, 5'
 580 CCGGGCTAAAGCCCTCCGG CTTTTT 3'. Thus, *sdaB* appears to be a single gene
 581 operon (Figure 9B).

582 **Analysis of SdaA and SdaB.** The *sdaA* and *sdaB* genes encode proteins of
 583 48.9 kD and 49.2 kD, respectively (Table 2). The hydrophobicity plots of the
 584 proteins suggest the presence of very hydrophobic regions (Figures 10 and 11).
 585 SdaA and SdaB exhibit four and five regions of 20 or more hydrophobic amino
 586 acids between amino acids 275 – 375 and 275 - 425, respectively. The
 587 hydrophobic nature of these amino acid segments may render them membrane
 588 spanning domains, or more likely constituents of the stabilizing hydrophobic core
 589 of cytoplasmic L-SD.

590 The *P. aeruginosa* L-SDs were compared with each other and other L-SD
 591 homologs (Table 3). The two *P. aeruginosa* L-SDs, SdaA (EC 4.2.1.13) and SdaB
 592 (EC 4.2.1.13), share 62 % homology with one another. *P. aeruginosa* SdaA and
 593 SdaB share greater than 41.9 % and 44.4 % homology, respectively, with all other
 594 L-SDs analyzed in this study. The least homology was seen with *S. pyogenes* SdaA

(12 % with SdaA and 11.1 % with SdaB). *P. aeruginosa* SdaA also shares 51.3 % and 51.6 % homology with *E. coli* SdaA and SdaB, respectively (Table 3). *P. aeruginosa* SdaB shares 51.8 % and 52.5 % homology with *E. coli* SdaA and SdaB, respectively (Table 3). Both SdaA and SdaB show high homology to L-SDs from *Mycobacteria*. When clustal method analysis was used to analyze the relative similarity of the L-SD enzymes the two *P. aeruginosa* and the two *Mycobacterium* enzymes were most closely related to their congeners (Figure 12). The *E. coli* and *Y. pestis* enzymes were most closely related to each other. *S. pyogenes*, the only Gram positive bacterium in the study was used on the outgroup (Figure 12).

Minimal Plating Experiments. Strains PAO1, PAOsdA, and PAOsdB were plated on NIV minimal media with various carbon sources: glucose (NIV-Glu), serine (NIV-Ser), glycine (NIV-Gly), leucine (NIV-Leu), serine-glycine (NIV-Ser-Gly), serine-leucine (NIV-Ser-Leu), serine-glycine-leucine (NIV-Ser-Gly-Leu). As controls the cultures were plated on NIV and NMM media. Both the number of the colonies and the characteristics were observed. The PAO1 colonies grew best in NIV-Glu (the positive control) media with the colony size recorded at 4.0 mm (Table 4). These colonies were large, healthy, pronounced, and harbored a large volume of cells per colony. As expected no colonies were observed in NMM media that served as our negative control. We expected no growth in the NIV plate that is also considered minimal media, but contains the amino acids isoleucine and valine. However, there were colonies, which were small (1 mm) and translucent with 91 % survival (Table 4). Similar translucent colonies were also observed on NIV-Ser (Table 4). The colony size on NIV-Ser-Leu was also 1 mm but the colonies were tight, compact, much more pronounced, and the amount of

619 cells/ colony was visibly greater (10x) than that of the PAO1 cells grown on NIV
620 media alone and at least five-fold more than NIV-Ser media. A significant
621 decrease in colony % survival (78 %) was noted for PAO1 colonies in NIV-Ser
622 media (Table 4). An even greater significant change in colony survival (63 %) was
623 observed in NIV-Ser-Leu media (Table 4).

624 The number of the colonies in NIV-Ser-Gly was similar to NIV-Glu but the
625 colony characteristics were distinctly different. The colonies on NIV-Gly media
626 (2.5 mm) were translucent with visibly fewer cells per colony than in NIV-Ser-Gly
627 (Table 4). The estimated amount of cells/ colony in NIV-Gly is 1/10th the NIV-Glu.

628 The best growth was observed in NIV-Ser-Gly-Leu with colonies slightly
629 smaller (3.5 mm) compared to those in NIV-Glu media (Table 4). The percentage
630 of PAO1 colonies that formed on NIV-Ser-Gly-Leu and NIV-Ser-Gly plate media as
631 compared to those that formed on NIV-Glu media is ~ 89 suggesting failure of 10
632 % of colonies to grow on this media (Table 4).

633 **PAOsdA Colony Description.** The mutant strain PAOsdA shared almost
634 similar percentage survival as the wild type PAO1. However, the colony size is
635 smaller in all the media tested (Table 4). PAOsdA exhibited similar growth
636 properties as compared with PAO1 in NIV-Glu media. The *sdaA* mutant also does
637 very poorly on NIV-Ser (Table 4).

638 The percentage survival in NIV-Ser-Gly-Leu was comparable to that for
639 PAO1 but the colonies were tight, compact, and healthy (2.5 mm). The only
640 observable difference was the decrease in colony size, attributable of course to
641 the absence of one of the L-SD encoding genes (*sdaA*). A significant decrease

in colony size was also observed when PAOsdA cells grown in NIV-Ser-Gly (1.5 mm), NIV-Ser-Leu (0.75 mm) and NIV-Ser (0.5 mm) as compared to PAO1 (Table 4).

The presence of trace amounts of leucine appears to enhance the growth of strain PAOsdA. The survival for strains growing on NIV-Ser-Leu (78 %) and NIV-Leu (74 %) were significantly greater than PAO1 cells grown on NIV-Ser-Leu (63.33 %) and NIV-Leu (73.78 %) (Table 4).

PAOsdB Colony Description. The mutant strain PAOsdB showed similar colony morphology as that of parent strain PAO1 (Table 4). There is an overall decrease in percentage survival in all the media tested.

A noticeable reduction was observed in NIV-Ser-Gly-Leu (61.48 %) (Table 4). The colonies on this media were also tight, compact, and healthy. Of significant note is the fact that unlike PAOsdA colonies that appeared to benefit slightly by leucine supplementation of NIV-Ser media, no increase in colony size was observed between PAOsdB grown on NIV-Ser-Gly-Leu (3.5 mm) versus those grown on NIV-Ser-Gly (3.5 mm) (Table 4).

Similar colony size was observed when PAOsdB colonies were grown on NIV-Ser and NIV-Ser-Leu. However, a significant decreased percentage survival was observed in NIV-Ser-Leu media versus NIV-Ser media (48 % vs 71 %) and also in NIV-Ser-Gly-Leu versus NIV-Ser-Gly media (61 % vs 81 %) (Table 4). The presence of trace amounts of leucine appears to inhibit growth of strain PAOsdB.

Growth Curve Properties of PAO1. *P. aeruginosa* PAO1 growth in rich liquid media and in minimal media supplemented with various amino acids was analyzed. From Figure 13 the growth curves, the doubling time was calculated. As expected PAO1 growth was optimal in rich LB media (Figure 13) with a doubling time of approximately 37.4 minutes (Figure 13, Table 5). PAO1 was also able to grow in NIV-Glu minimal media with a doubling time of 80.5 min. In the presence of Ser-Gly and Ser-Gly-Leu the growth rate was drastically reduced with a doubling time change of 285 and 210 min, respectively (Figure 13, Table 5). PAO1 was unable to utilize serine and leucine as the sole carbon source (Figure 13).

Growth Properties of *sdaA* and *sdaB* Mutants. The growth properties of the L-SD mutants, PAO*sdaA* and PAO*sdaB* were compared with the parental PAO1. The growth properties of PAO*sdaA* and PAO*sdaB* did not differ significantly from PAO1 in LB media (Figure 14A, Table 5) and NIV-Glu (Figure 14B, Table 5). Similar to PAO1, the mutants were able to grow on Ser-Gly-Leu, however the doubling time was slightly reduced suggesting a slight increase in growth rate (Figure 14F, Table 5). The growth of both *sdaA* and *sdaB* mutants was significantly compromised in NIV-Ser-Gly media that lacked leucine. Where PAO1 growth doubled at 285 min, the *sdaA* and *sdaB* mutants doubling times were significantly longer, 812 and 682 min, respectively (Figure 14E, Table 5).

As expected, similar to the prototype strain PAO1, both PAO*sdaA* and PAO*sdaB* mutants failed to grow in NIV with serine as the sole carbon source (Figure 14C, Table 5). In fact, the addition of leucine (Figure 14D, Table 5) to NIV-

686 Ser failed to support growth. However, addition of glycine to NIV-Ser media did
687 promote growth of both mutants (Figure 14E, Table 5).

688 ***P. aeruginosa* L-SD Activity.** The L-SD enzyme assay was performed on
689 strain PAO1 grown in NIV-Glu-Gly and NIV-Ser-Gly-Leu. In both media, there is a
690 lag of 20-30 min, followed by a quick burst of activity that plateaus at 80 min
691 (Figure 15). The L-SD activity rate in Ser-Gly-Leu (0.155 $\mu\text{g}/\text{min}$) is twofold higher
692 than the activity rate in NIV-Glu-Gly media (0.076 $\mu\text{g}/\text{min}$).

693 After determining the time of maximal activity, subsequent assays were
694 done on 14 hr cultures, and the amount of pyruvate made was determined after
695 50 min. The L-SD activity of PAO1 in NIV-Ser-Gly-Leu, NIV-Glu-Gly, NIV-Glu, and
696 NIV-Gly-Leu were analyzed (Table 6). There is a significant 28.6 % decrease in L-
697 SD activity upon addition of leucine to the NIV-Glu media (Table 6). In contrast, a
698 3.2-fold increase in L-SD activity was observed in NIV-Ser-Gly-Leu when
699 compared to NIV-Glu-Gly media (Table 6). No significant difference in L-SD
700 activity was observed in PAO1 grown in NIV-Gly-Glu media or NIV-Glu media
701 (Table 6).

702 When strain PAOsdA was grown in the same media the same general
703 pattern of induction was observed, however the amount of pyruvate produced
704 is generally lower than the parent PAO1 (Table 6). No observable increase in
705 activity was observed when glycine was added to NIV-Glu media (Table 6). A 44
706 % decrease in activity was observed when leucine was added to NIV-Glu media
707 and a 2.9-fold increase in L-SD activity was observed between strains grown in
708 NIV-Ser-Gly-Leu as compared to NIV-Glu media (Table 6).

709 In PAOsdB a decreased pyruvate production was observed as
 710 compared to PAO1 in NIV-Glu, NIV-Ser-Gly-Leu, and NIV-Gly-Leu media (Table 6).
 711 However, in NIV-Glu-Gly media PAOsdB and PAO1 showed indistinguishable L-
 712 SD activity. A 1.4-fold increase in activity was observed when glycine was added
 713 to NIV-Glu media (Table 6). A 1.6-fold increase in L-SD activity was observed in
 714 Ser-Gly-Leu media as compared to NIV-Glu media and a 53 % decrease in L-SD
 715 activity was observed upon leucine supplementation (Table 6).

716 **Serine Growth Mutants Characteristics.** After growth for 36 hours the OD₆₀₀
 717 values for the five serine utilizing mutants (*ser1* to *ser5*) were recorded and their L-
 718 SD activity was assayed (Table 7). Unlike the parent PAO1, *ser1*, *ser2*, *ser3*, *ser4*
 719 and *ser5* were able to utilize serine as a sole carbon source (Table 7).

720

721 DISCUSSION

722 All pathogens require high energetic influxes to counterattack the host
723 immune system and without this energy bacterial infections are easily cleared.
724 This study was an investigation into a highly bioenergetic pathway in
725 *Pseudomonas aeruginosa* involving the amino acid L-serine and the enzyme L-
726 serine deaminase (L-SD). Recent evidence linked L-SD directly to the
727 pathogenicity of several organisms including but not limited to *Campylobacter*
728 *jejuni*, *Mycobacterium bovis*, *Streptococcus pyogenes*, and *Yersinia pestis*
729 (Velayudhan *et al.*, 2004). We hypothesized that *P. aeruginosa* L-SD is likely to be
730 critical for its virulence. We analyzed the ability of *P. aeruginosa* to utilize serine
731 and the role of SdaA and SdaB in serine deamination by comparing mutant
732 strains of *sdaA* (PAO*sdaA*) and *sdaB* (PAO*sdaB*) with their isogenic parent *P.*
733 *aeruginosa* PAO1.

734 ***P. aeruginosa* Harbors Two L-SDs.** *P. aeruginosa* Genome sequencing
735 revealed the presence of two genes encoding L-SDs, *sdaA* and *sdaB* (PA2443
736 and PA5379) (Stover *et al.*, 2000). These two *P. aeruginosa* L-SDs, SdaA and SdaB
737 share 62 % homology to one another (Table 3). The high homology suggests that
738 one of these two genes became incorporated into the genome via duplication
739 of the existing *sdaA* or *sdaB*. The dissimilarity between these two proteins is also
740 large enough to suggest that the duplication event took place early in the
741 evolution of *P. aeruginosa*.

742 Multi-copy genes are usually essential genes, encoding a product that the
743 bacterium cannot afford to lose. They offer protection against mutation and loss
744 of phenotype. L-SD is usually encoded multiple times within a bacterial genome.

745 *P. aeruginosa* harbors two genes encoding L-SD (Stover *et al.*, 2000). *E. coli* bears
746 three copies of L-SD in its genome (Keseler *et al.*, 2005). The presence of multiple
747 copies of this enzyme in bacterial genomes adds weight to the hypothesis that L-
748 SD is important for growth of a bacterium.

749 In *E. coli* and *P. aeruginosa*, each multicopy L-SD was proven to be
750 functional and yet both bacteria exhibited growth failure with serine as the sole
751 carbon source (Alfoldi & Rasko, 1970). Though the enzyme is encoded multiple
752 times in the genome and its expression should be high within the constitutive cell,
753 no growth is observed in *E. coli* unless leucine is added to the media, and no
754 growth is observed in *P. aeruginosa* unless glycine is added to the media
755 (Newman *et al.*, 1985)(Table 4, 5). In *E. coli* it is known that L-SD is kept under tight
756 negative regulation by the Lrp protein and when leucine is added this negative
757 inhibition is released and growth is allowed (Newman *et al.*, 1985). Although an
758 Lrp homolog is present in the *P. aeruginosa* genome, the opposite phenotypic
759 effect is observed when leucine is added to the media (Table 4, 5, 6).

760 The role of glycine in L-SD induction is novel in the bacterial literature. In
761 subsequent sections, the possibility of glycine enhancing transport of L-serine into
762 the cell is discussed as a possible explanation of the induction potential of
763 glycine on L-SD expression.

764 ***P. aeruginosa* L-SDs are Nonessential.** The loss of *sdaA* and *sdaB* had no
765 effect on *P. aeruginosa* growth in LB media (Figure 14A). This is not surprising as LB
766 contains carbon sources in the media other than serine that allow growth and
767 therefore *sdaA* and *sdaB* were not essential. In addition, the absence of the L-SD

768 encoding genes did not affect growth in glucose minimal media (Figure 14B).
 769 This also makes sense given the fact that the carbon source utilized for growth in
 770 this media is glucose and not L-serine. In fact, as expected, the L-SD activity in LB
 771 is lower than in minimal medium with glucose (Table 6). It is possible that the use
 772 of serine becomes critical at stationary phase when the nutrients in the media
 773 have been exhausted. The assay was performed after 14 hours of growth where
 774 in rich LB media the culture has passed the stationary phase and has entered the
 775 death phase (Figure 14A). It is possible that at this stage of growth, PAO1 L-SD
 776 activity will be reduced, especially if as in other organisms L-serine is the first
 777 amino acid utilized (Prüß, 1994). Thus, a detailed time course experiment is
 778 required to determine the L-SD activity.

779 ***P. aeruginosa* cannot utilize Serine as a Sole Carbon Source.** PAO1 colony
 780 size on the minimal plating experiments (Table 4), suggests that *P. aeruginosa* is
 781 not capable of growth with serine as a sole carbon source This finding is further
 782 confirmed by growth curves (Figure 14C). Like the prototype strain, PAO1, no
 783 growth was observed in both the *sdaA* and *sdaB* mutant strains in media with L-
 784 serine as the sole carbon source (Figure 14C). *E. coli* also exhibits no growth on
 785 serine as the sole carbon source, but as stated earlier the enzymes, though
 786 functional, are held under tight negative Lrp regulation.

787 In this study, we have also isolated five serine growth mutants (Table 7)
 788 that now can utilize serine as the sole carbon source. Likely these strains have
 789 mutations that either up regulate an activator or have inactivated a repressor. In
 790 either case, the net result is constitutive expression of L-SD allowing the bacteria

791 to utilize serine. Future mapping of the mutations in these strains will likely reveal
792 the intricate mechanisms involved in regulating the expression of L-SDs.

793 **Both SdaA and SdaB Contribute to the Total L-SD Activity.** The similarity
794 between both *P. aeruginosa* L-SDs and the L-SDs of *C. jejuni*, *M. bovis*, *Y. pestis*,
795 and *E. coli* suggests that these enzymes work through a similar mechanism in
796 these bacteria and likely have a similar function (Table 3). Our analysis of
797 PAOsdA and PAOsdB mutants show that both SdaA and SdaB contribute to
798 the total L-SD activity (Table 6). The loss of either *sdaA* or *sdaB* in serine-glycine
799 media contributed to a strikingly large increase in doubling time as compared to
800 PAO1, suggesting again that both SdaA and SdaB are contributing to PAO1
801 growth in glycine supplemented serine minimal media (Table 5). Based on the
802 ability of the mutants to convert serine to pyruvate, we estimated that SdaA and
803 SdaB contribute 34 % and 66 % of the total activity, respectively (Table 6). Hence,
804 loss of one gene is unable to abolish the activity completely. Since SdaB appears
805 to be the major contributing L-SD, we predict that PAOsdB is likely to be less
806 virulent as compared to PAOsdA.

807 **Glycine Enhances *P. aeruginosa* Serine Utilization.** *P. aeruginosa* cannot
808 grow on serine minimal media alone (Figure 14C). However, the addition of
809 glycine resulted in a colony size similar to the one obtained with glucose media.
810 *P. aeruginosa* is also capable of growth in minimal media with glycine as the sole
811 carbon source (Table 4). However, the extent of glycine utilization was minimal in
812 comparison to PAO1 growth in serine media supplemented with glycine (Table
813 4). The glycine-dependent growth in serine media can also be seen in the

814 growth curve (Figure 14E). These data suggest an ability of L-glycine to enhance
815 L-serine utilization in *P. aeruginosa* PAO1. This is supported by the total L-SD
816 activity where the activity was elevated in media with serine and glycine as
817 compared to growth in glucose minimal media alone (Table 6).

818 The analyses of the *sdaA* and *sdaB* mutant strains suggest that the SdaA L-
819 SD activity is glycine-dependent (Table 6). The prototypic PAO1 that harbors
820 active SdaA and SdaB showed a 2.8-fold induction in L-SD activity upon addition
821 of glycine to serine media (Table 6). Strain PAO*sdaB* that has an active SdaA
822 exhibited a similar induction (2.7- fold) in the presence of L-serine (Table 6). In
823 contrast, PAO*sdaB* that harbors active SdaA showed no significant induction of
824 L-SD activity upon addition of L-serine (Table 6). Interestingly, glycine induction of
825 L-SD activity in glucose media was observed in strain PAO*sdaB* (Table 6). No
826 effect was observed in PAO1 or in PAO*sdaA* under the same experimental
827 conditions (Table 6).

828 It is not known how L-serine enters the *P. aeruginosa* cell. Genomic
829 analysis reveals the absence of specific L-serine membrane transporters (Stover
830 *et al.*, 2000). We speculate that an L-serine / L-glycine symporter is present within
831 the *P. aeruginosa* outer membrane which allows L-serine entrance into the cell
832 only in the presence of L-glycine. If this were the case then in the presence of
833 glycine, serine would enter the cell and induce transcription of the L-SD enzymes.
834 Indeed 4-fold induction of L-SD activity was observed when glycine and serine
835 were present in the media (Table 6). If glycine were not present, then serine
836 would not be able to enter the cell, L-SD expression would not be induced, and
837 L-SD activity would remain constitutive. Indeed, no significant difference in L-SD

838 activity was observed upon addition of glycine to glucose minimal media (Table
839 6).

840 **Serine is the Inducer of *P. aeruginosa* L-SD Activity.** Our data also shows
841 that glycine alone cannot induce the L-SD activity as there was no significant
842 increase in L-SD activity between PAO1 grown in glucose and glucose-glycine
843 media (Table 6). Thus, serine has to be present for the induction of L-SD activity. A
844 3.2-fold increase in total pyruvate production was observed between strain
845 PAO1 grown in serine-glycine as compared to glucose media (Table 6). This
846 strongly argues that L-serine itself is an inducer of L-SD activity. Interestingly, there
847 is a slight increase of SdaB activity in glucose-glycine media in the absence of
848 serine. This might just be a basal activation of *sdaB* expression but full induction
849 requires the inducer serine. The fact that L-serine is an inducer of L-SD makes
850 biological sense. It is bio energetically inefficient to transcribe a gene, and
851 translate a protein, if that protein is not going to be used within the cell.

852 **Leucine Inhibits *P. aeruginosa* L-SD Activity.** In *E. coli* the addition of L-
853 leucine to serine minimal media induces the production of L-SD and the
854 concomitant breakdown of L-serine for energy requirements (Newman *et al.*,
855 1985). This induction takes place via regulation of the leucine-responsive
856 regulatory protein **Lrp** which negatively regulates *E. coli* *sda* genes (Calvo &
857 Matthews, 1994; Newman & Lin, 1995). *P. aeruginosa* genome analysis revealed
858 the presence of an *E. coli* Lrp homolog (PA5308)}; Stover *et al.*, 2000). We
859 hypothesized that L-SD regulation in *P. aeruginosa* occurred via the same
860 mechanism.

Supplementing serine with L-leucine both in the minimal plates (Table 4) and liquid media (Figure 14D) failed to support *P. aeruginosa* growth. In contrast, a slight but significant decrease in doubling time (that is increased growth rate) was observed in NIV media supplemented with serine-glycine-leucine as compared to serine-glycine alone (Table 5). This increased growth rate may be due to leucine and not serine catabolism since growth in minimal media with leucine as the sole carbon source was observed (Table 4). In addition, the small differences in the doubling time between PAO1 grown in serine-glycine-leucine with serine-glycine explain why colonies on minimal media plates with similar amino acid combinations appeared indistinguishable (Table 4).

Analysis of the whole cell L-SD assays revealed a 29 % decrease in L-SD activity upon L-leucine addition to glucose media (Table 6). It appears that not only does L-leucine fail to contribute to L-serine deamination in *P. aeruginosa* but it down regulates the total L-SD activity. In fact, both SdaA and SdaB activity were inhibited by the addition of L-leucine to glucose minimal media (Table 6). The relative rates of leucine inhibition showed a slightly greater inhibitory effect on SdaA (53.0 %) than SdaB (44 %) (Table 6). This is in sharp contrast to the L-SD regulatory system in *E. coli*, where leucine interaction with the Lrp protein induces L-SD activity (Newman & Lin, 1995). Thus, it appears that leucine-dependent regulation of *P. aeruginosa* L-SD activity is Lrp-independent.

Infectivity Study. The hypothesis of this study is that *in vivo* utilization of the amino acid L-serine confers a highly efficient bioenergetic pathway to *P. aeruginosa*, which is utilized to counterattack host immune defense. Since both

884 SdaA and SdaB contribute to the total L-SD activity, a double mutant is required
 885 to determine the role of serine deamination in pathogenicity. In addition, the
 886 strain *P. aeruginosa* PAO1 is less virulent as compared to *P. aeruginosa* PA14. The
 887 second half of this study focused on construction of suicide vectors harboring
 888 insertional inactivated gene cassettes of *sdaA* and *sdaB*. The suicide vectors are
 889 designed to lack a compatible origin of replication with *P. aeruginosa* (Schweizer
 890 & Hoang, 1995). This characteristic forces them to integrate into the *P.*
 891 *aeruginosa* chromosome at regions of homology. Since, the suicide vectors
 892 constructed in this study bear the *sdaA::aacCI*, *sdaB::aacCI*, and *sdaB::aphI*
 893 cassettes, it is possible to selectively mutate the *sdaA* and *sdaB* genes in *P.*
 894 *aeruginosa* strains of interest including PA14 (Schweizer & Hoang, 1995).

895 Subsequent investigations into the role of L-SD in *P. aeruginosa*
 896 pathogenicity will require animal model infectivity studies. Aspects of innate
 897 immunity are evolutionarily conserved amongst vertebrates, invertebrates, and
 898 plants (Kurz & Ewbank, 2003). In response, the evolution of vertebrate,
 899 invertebrate, and plant pathogens has followed a similar trend. The result is that
 900 bacterial pathogenic mechanisms which are utilized to invade invertebrate
 901 hosts, will serve the same or similar functions in vertebrate and plant hosts and
 902 genes essential for virulence in invertebrates prove to be essential for virulence in
 903 other biological kingdoms (Tan *et al.*, 1999b; Gan *et al.*, 2002; Kothe *et al.*, 2003;
 904 Aballay *et al.*, 2000; Garsin *et al.*, 2001; Sifri *et al.*, 2003; Jansen *et al.*, 2002). To
 905 study the *in vivo* characteristics of *P. aeruginosa*, several model systems may be
 906 employed. Apart from the murine model system, the most commonly used are
 907 the *Arabidopsis thaliana*, *Drosophila melanogaster*, and *Caenorhabditis*

908 *elegans* infectivity model systems (Rahme *et al.*, 1995; Vodovar *et al.*, 2004; Tan
909 *et al.*, 1999a) The *C. elegans*-*P. aeruginosa* pathogenicity model system is the
910 simplest model system to study bacterial pathogenicity.

911 *C. elegans* is a nematode which exhibits a three-day life span and
912 reaches 1.5 mm in length when fully grown (Brenner, 1974; Byerly *et al.*, 1976). It
913 contains rudimentary digestive, nervous, muscle, and reproductive systems
914 (Brenner, 1974). It is entirely transparent and the fate of each of its adult somatic
915 cells has been mapped. *C. elegans* is ubiquitous in the soil, where it feeds on
916 various bacteria in its surroundings. In the lab it is routinely cultured on lawns of
917 the bacterium *E. coli* strain OP50, but if given no alternative, it will eat whichever
918 bacterium is provided for it (Lewis & Fleming, 1995). When fed *P. aeruginosa*
919 Strain PA14, the worms acquire a gut infection, leading to reduced viability of
920 the worms (Tan *et al.*, 1999a). This model system is especially useful for our
921 purposes for two reasons. The first is that the severity of the infection correlates to
922 the virulence of the bacterium (Tan *et al.*, 1999b). The second is that observed
923 variations from the prototype strain by isogenic mutants reveal the contribution
924 of individual genes to the virulence of *P. aeruginosa* (Ausubel, 2005). When fed *P.*
925 *aeruginosa* strain PAO1, worms are killed via a distinct toxicity mechanism
926 involving the production of cyanide and other virulence factors (Gallagher &
927 Manoil, 2001). This worm-killing assay is useful in determining variations in virulence
928 factor production among *P. aeruginosa* strains. The mutant strains (PAOsdA
929 and PAOsdB) utilized in this study are derived from the prototype strain PAO1.
930 This strain is not sufficiently virulent to establish an infection in the gut of *C.*
931 *elegans* (Tan *et al.*, 1999a). *P. aeruginosa* PA14 shows increased virulence as

932 compared to strain PAO1 (Tan *et al.*, 1999a) as it is capable of forming a
933 persistent infection in the gut of *C. elegans* (Mahajan-Miklos *et al.*, 1999). The
934 PA14-derived L-SD mutants will facilitate further elucidation of the role of L-SD in
935 *P. aeruginosa* metabolism and pathogenicity.

936 ***P. aeruginosa* L-SD and its role in pathogenesis.** A pathogenic organism
937 such as *P. aeruginosa* needs to obtain energy from host metabolites. Given the
938 selective advantage conferred on organisms with high L-SD activity *in vitro*, the
939 ability to utilize L-serine released by proteolytic cleavage should confer a
940 selective advantage on these pathogenic organisms *in vivo* (Zinser & Kolter,
941 2000). This study showed the ability of *P. aeruginosa* to break down the amino
942 acid L-serine via two enzymes SdaA and SdaB (Table 7). The induction of L-SD
943 activity requires L-glycine (Table 7). *In vivo* and during infection, *P. aeruginosa* will
944 be surrounded by a large enough concentration of amino acids including
945 glycine which will induce the activity of L-SD. This increased L-SD activity will be
946 utilized by *P. aeruginosa* to obtain energy. The contribution to L-SD activity by
947 SdaA and SdaB will likely depend on the ratio of glycine, serine and leucine in
948 the surrounding tissues, because differential induction intensities were observed
949 between the two L-SD enzymes (Table 7).

950 In the presence of so many other carbon sources, why should the
951 breakdown of one amino acid prove to be so important? L-SD was proven to be
952 essential for infectivity by *C. jejuni* and *M. bovis* and thus it may also be required
953 for *P. aeruginosa* infectivity (Velayudhan *et al.*, 2004; Chen *et al.*, 2003). *P.*
954 *aeruginosa*, is known for its metabolic diversity, with a capacity to utilize over 200
955 different carbon sources for energy (Vasil, 1986). It has a vast amount of

956 metabolic genes and metabolic regulation assisting in its energy acquisition
 957 capabilities (Vasil, 1986). If further investigation, proves L-SD to be important for
 958 pathogenicity in *P. aeruginosa*, then perhaps this enzyme is important across the
 959 bacterial kingdom. Furthermore, if L-SD is important for pathogenicity an innate
 960 property of prokaryotic L-SD (4FeS), could allow for selective attack on this
 961 enzyme while leaving the eukaryotic homolog unhindered (Cicchillo *et al.*, 2004).

962 In conclusion this study is the first attempt to characterize the highly
 963 bioenergetic L-serine deaminase pathway in *P. aeruginosa*. We demonstrate the
 964 both *P. aeruginosa* L-SDs, SdaA and SdaB contribute to L-serine deamination. The
 965 amino acids glycine and serine were shown to induce L-SD activity *in vivo*. In
 966 sharp contrast to *E. coli* L-SD regulation, the amino acid L-leucine was shown to
 967 inhibit L-SD activity. Lastly, suicide vectors were constructed which allow for
 968 selective mutation of the *sdaA* and *sdaB* genes on any *P. aeruginosa* strain of
 969 interest.

970 Microbiologists have long looked for the “silver bullet” to attack
 971 microorganisms. As a result, the bioenergetics of infection has been overlooked
 972 as a possible source of therapeutic targets. In this regard, the bioenergetics of
 973 infection holds great promise in the dynamic interactions between host and
 974 pathogen. Against an ever-evolving microbe, it is imperative to seek alternate
 975 modes of attack. *In the absence of energy, there is no life.*

976

977

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987

988

989 **CONFLICTS OF INTEREST**

990 The authors declare that there no conflicts of interest.

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1211 **Figures**

1212 **Figure 1. L-Serine catabolism.** L-serine is converted to pyruvate via a one-step
1213 reaction catalyzed by L-serine deaminase (L-SD). One molecule of H₂O is
1214 absorbed and one molecule of NH₃ is released during the process. Pyruvate then
1215 enters the citric acid cycle yielding one GTP, four NADH₂, and one FADH₂
1216 molecule.

1217 **Figure 2. Comparison of L-serine deamination with glycolysis.** In comparison to
1218 glycolysis which involves ten enzymes ultimately leading to the production of 38
1219 ATPs, L-serine catabolism only requires one enzyme, L-serine deaminase (L-SD)
1220 ultimately leading to the production of 15 ATPs. There is a 15:1 ATP to enzyme
1221 ratio for L-serine catabolism as compared to a 3.8:1 ATP to enzyme ratio in
1222 glycolysis.

1223 **Figure 3. Construction of a suicide vector containing insertional inactivated *sdaA***
1224 **with *aacCI*.** The *sdaA* gene was PCR amplified from the PAO1 chromosome and
1225 annealed to pCR2.1 forming pCSMLA. The *SmaI* fragment from pUCGm
1226 containing *aacI* was cloned into a unique *SmaI* site on the *sdaA* gene on
1227 pCSMLA forming pCSMLA2. A 3,117-bp *HindIII*-*XbaI*-Klenow treated fragment
1228 containing the *sdaA::aacCI* fragment was sub cloned into a *SmaI* site on the
1229 suicide vector pEX100T that contains *sacB* gene for counter selection and
1230 named pXSMLA.

1231 **Figure 4. Construction of a suicide vector containing insertional inactivated *sdaB***
1232 **with *aphI*.** The *sdaB* gene was PCR amplified from the PAO1 chromosome and
1233 annealed to the vector pCR2.1 forming pCSMLB. An *EcoRI* fragment containing
1234 the *sdaB* gene from pCSMLB was sub cloned into at the *EcoRI* site on pUC19
1235 forming pUSMLB. The 979-bp *StuI* digested *aphI* PCR amplicon from EZ-KM
1236 transposon was ligated to *StuI* digested pUSMLB forming pUSMLBK. The 3010-bp
1237 *EcoRI*- Klenow treated *sdaB::aphI* from pUSMLBK was sub cloned into the *SmaI*
1238 site of the suicide vector pEX100T that contains *sacB* gene for counter selection
1239 forming pXSMLBK.

Figure 5. Construction of a suicide vector containing insertional inactivated *sdaB* with *aacCI*. The 855-bp *Sma*I fragment containing *aacCI* cassette from pUCGm was ligated to *Stu*I digested pUSMLB (see Figure 6) forming pUSMLBG. The *Eco*R1-Klenow treated *sdaB*::*aacCI* from pUSMLBG was sub cloned into the *Sma*I site of pEX100T forming pXSMLBG.

Figure 6. Construction of complementing plasmids. In order to obtain plasmids harboring *sdaA* and *sdaB* that are efficiently maintained for phenotypic complementation in *P. aeruginosa*, the broad host-range plasmid pME6030 was used. *Hind*III -cut pME6030 was ligated a 2250-bp *Hind*III fragment harboring *sdaA* from pCSMLAH3 forming pMEsdaA. *Eco*R1-cut pME6030 was ligated to a 2049-bp *Eco*R1 fragment harboring *sdaB* from pUSMLB (Figure 6) generating pMEsdaB.

Figure 7. Verification of *sdaA* plasmids and complementing plasmids constructed using 1.0 % agarose electrophoresis. Lanes 1, DNA Ladder N3232S (New England Bio labs); Panel A) 2. *sdaA* gene amplified from the PAO1 chromosome using the primers PAOsdaAH3F and PAOsdaAH3R (2346bp); 3. *Hind*III- cut pCSMLAH3 yielding three fragments of 67, 2250, and 3882 bp; 4. 855-bp *aacI* from pUCGm; 5. *Sma*I-cut pCSMLA (6199 bp); 6. *Hind* III-*Xba*I-cut pCSMLA2 yielding two 3211 and 3819 bp fragments; 7. 3211 bp *sdaA*::*aacI* from pCSMLA2; 8. *Sma*I- cut pEX100T (5846 bp); 9. *Fsp*I-cut pXSMLA yielding two confirmatory linear DNA 4183 bp and 4874 bp fragments. Panel B) 2. 8021-bp *Eco*R1- cut pME6030; 3. 8021-bp *Hind*III-cut pME6030; 4. 2049-bp *Eco*R1 fragment with *sdaB* from pUSMLB; 5. 2250-bp *Hind*III fragment with *sdaA* from pCSMLAH3; 6. *Eco*R1-cut pMEsdaB yielding two fragments of 8021 bp and 2049 bp; 7. *Hind*III-cut pMEsdaA yielding two fragments of 8021 bp and 2250 bp.

Figure 8. Verification of *sdaB* plasmids constructed using 1.0 % agarose electrophoresis. Lanes 1. DNA Ladder N3232S (New England Bio labs); 2. *sdaB* gene amplified from the PAO1 chromosome using the primers PAOsdaBF and PAOsdaBR (2043 bp); 3. *Eco*R1-cut pCSMLB yielding two fragments of 2049 and 3913 bp; 4. 2049-bp *Eco*R1 from pCSMLB with *sdaB*; 5. *Eco*R1-cut pUSMLB, yielding

the expected 2049 and 2686 bp fragments; **6.** 2049-bp *EcoRI* fragment harboring *sdaB* from pUSMLB; **7.** 979-bp amplicon from EZ-transposon kit harboring *aphI*; **8.** 855-bp *aacCI* amplicon from pUCGm, **9.** *StuI*- cut pUSMLB (4735 bp); **10.** *EcoRI*-cut pUSMLBK yielding two fragments of 2686 bp and 3010 bp; **11.** *EcoRI*-cut pUSMLBG yielding two fragments of 2686 and 2904 bp; **12.** 3010-bp fragment from pUSMLBK with *sdaB::aphI*; **13.** *sdaB::aacCI* from pUSMLBG; **14.** *FspI*-cut pXSMLBK yielding four fragments of 363, 1023, 1714, and 2596 bp; and **15.** *FspI*-cut pXSMLBG yielding four fragments of 363, 1023, 1608, and 2596 bp.

Figure 9. Chromosomal location of *P. aeruginosa* *sda* genes. *sdaA* and *sdaB*. **(A).** The *sdaA* (PA2443) gene is found at position 2742353 to 2740977 within the *P. aeruginosa* Strain PAO1 chromosome (<http://v2.pseudomonas.com/getAnnotation.do?locusID=PA2443>). **(B).** *P. aeruginosa* SdaB is encoded by *sdaB* (PA5379) found at position 6056877 to 6058253 within the PAO1 chromosome (<http://v2.pseudomonas.com/getAnnotation.do?locusID=PA5379>).

Figure 10. SdaA protein analysis. The amino acid sequence of SdaA **(A)** was analyzed via a Kyte Doolittle Hydrophobicity plot **(B)** ran by Colorado State University <http://www.vivo.colostate.edu/molkit/hydropathy/index.htm>.

Figure 11. SdaB protein analysis. The amino acid sequence of SdaB **(A)** was analyzed via a Kyte Doolittle Hydrophobicity plot **(B)** ran by Colorado State University <http://www.vivo.colostate.edu/molkit/hydropathy/index.htm>.

Figure 12. Clustal analysis of LSD homologs. This analysis revealed that the two *P. aeruginosa* L-SDs and the two *Mycobacterium* enzymes were most closely related. *E. coli* and *Y. pestis* enzymes were most closely related. *C. jejuni* is sister to these species and *S. pyogenes*, being the only Gram positive bacterium in the study is separated from all other species in the study

Figure 13. *P. aeruginosa* PAO1 growth in various media. PAO1 growth was optimal in rich LB media (●), NIV media with Glucose (◆), serine (■), serine and

1298 glycine (▲), serine and leucine (✕), serine, glycine and leucine (✕). The growth
1299 OD600 was determined for every hour for 15 hours.

1300 **Figure 14. Growth characteristics of *P. aeruginosa* *sda* mutants.** The growth
1301 pattern of PAO*sdaA* (■) and PAO*sdaB* (▲) were compared with their isogenic
1302 parent strain PAO1 (◆). The cells were cultured in (A) LB, (B) glucose, (C) NIV
1303 minimal media with serine, (D) NIV minimal media with serine and leucine, (E) NIV
1304 minimal media serine and glycine, and (F) NIV minimal media with serine, glycine
1305 and leucine.

1306 **Figure 15. The L-serine deaminase enzyme activity of *P. aeruginosa* PAO1.** The L-
1307 SD activity was measured after culturing the bacteria in NIV media with (A)
1308 glucose and glycine (NIV Glu-Gly) and, (B) serine, glycine and leucine (NIV Ser-
1309 Gly-Leu).

Table 1. Genotypes and source of bacterial and nematode strains, plasmids, and primers

Strains/Plasmids	Genotype	Reference/ Source
<i>Escherichia coli</i>		
DH5a	F ⁺ λ80dlacZλM15λ (lacZYA-argF) U169 deoR recA1 endA1 hsdR17(rk-gyrA96 thi-1 hsdR17 supE44 relA1 deoR Δ(lacZYA-argF) U169	NewEngland Biolabs
OP50	Uracil auxotroph, limited growth on NGM	(Brenner, 1974)
TOP10F'	F'(lacI ^q Tn10 (Tet ^R)) mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZ ΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str ^R) endA1 nupG	Invitrogen
MT102F-	thi araD139 ara-leu D7679 D(lacIOPZY) galUgal'K r ⁻ m ⁺ Smr	T. Hansen, Novo Nordisk A/S
<i>P. aeruginosa</i>		
PAO1	Nonmucoid prototype strain	(Holloway & Matsumoto, 1984)
PAOser1	PAO1 mutant that can utilize serine	This study
PAOser2	PAO1 mutant that can utilize serine	This study
PAOser3	PAO1 mutant that can utilize serine	This study
PAOser4	PAO1 mutant that can utilize serine	This study
PAOser5	PAO1 mutant that can utilize serine	This study
PAOsdaA	ΔsdaA (ISphoA/hah)1140	(Jacobs et al., 2003)
PAOsdaB	ΔsdaB (ISphoA/hah)473	(Jacobs et al., 2003)
PA14	Nonmucoid burn wound isolate	(Rahme et al., 1995)
<i>C. elegans</i>		
Bristol N2	Prototype Strain	(Brenner, 1974)
Plasmids		
pCR2.1	ColE1 oriV lacZα (Ap ^R Km ^R)	Invitrogen
pEX100T	Ap ^R ;sacB oriT	(Schweizer & Hoang, 1995)
pUCGM	Ap ^R ,Gm ^R ;pUC19 derivative containing gentamycin cassette	(Schweizer, 1993)
pUC19	Ap ^R , pBR322 derived	(Yanisch-Perron et al., 1985)
pME6030	Tc ^R ; oriVpVS1 oriVp15A oriT	(Heeb et al., 2000)
pRK2013	Km ^R oriColE1 RK2-Mob ⁺ RK2-Tra ⁺)	(Figurski & Helinski, 1979)
pRK600	ColE1 oriV; RP4tra ₊ RP4oriT; Cm ^R ; helper in triparental matings	(Kessler et al., 1992)
pCSMLA	pCR2.1 with 2,224bp fragment containing sdaA amplified with primers PAOsdaAF and PAOsdaBR	This study
pCSMLAH3	pCR2.1 with 2,250 bp fragment containing sdaA amplified with primers PAOsdaA-Hind3F and PAOsdaA-Hind3R	This study
pCSMLA2	pCSMLA with 855bp Sma1 aac1 cassette from pUCGM	This study
pXSMLA	pEX100T with 3117bp Hind3- Xba1 DNA from pCSMLAG	This study
pCSMLB	pCR2.1 with 2031 bp fragment containing sdaB amplified with primers PAOsdaBF and PAOsdaBR	This study
pUSMLB	pUC19 with 2049 bp fragment containing sdaB	This study

pUSMLBK	pUSMLB with 979 bp <i>aphI</i> cassette amplified using primers EZKM- <i>Stu</i> 1F and EZKM- <i>Stu</i> 1R	This study
pUSMLBG	pUSMLB with 855bp <i>Sma</i> 1 <i>aac1</i> cassette from pUCGM	This study
pXSMLBK	pEX100T with 2886 bp <i>Eco</i> R1 DNA from pUSMLBK	This study
pXSMLBG	pEX100T with 3010 bp <i>Eco</i> R1 DNA from pUSMLBG	This study
pMEsdaA	pME6030 with 2250 bp <i>Hind</i> III DNA from pCSMLAH3	This study
pMEsdaB	pME6030 with 2049 bp <i>Eco</i> R1 DNA from pUSMLB	This study

Primers

PAOsdaA F	CGTGGAGCATAAGCCGAAGATGAT	This study
PAOsdaA R	CGCGATCGATTGTCGGGAAAGAAA	This study
PAOsdaA H3F *	ATATAT <u>AAGCTI</u> CGTGGAGCATAAGCCGAAGATGAT	This study
PAOsdaA H3R *	ATAATT <u>AAGCTI</u> CGCGATCGATTGTCGGGAAAGAAA	This study
PAOsdaBF	TACCCGCTGTGTCGCATTCAATCA	This study
PAOsdaBR	ATTGTTGGAATTCCGGGTGGCTG	This study
EZ-KM <i>Stu</i> 1F *	ATATAT <u>AGGCCI</u> CATTGC ACAAGATAAAAA	This study
EZ-KM <i>Stu</i> 1R *	ATATAT <u>AGGCCI</u> C GCCGTCCCGTCAAG	This study

* The red under-lined lettering indicates recognition sequences for restriction enzymes

Table2. SdaA and SdaB protein analysis.

Analysis^a	SdaA*	SdaB*
Molecular Weight	48,945.34	49,217.49
Length	458	458
1 microgram = (picomoles)	20.431	20.318
Molar Extinction coefficient	29,790 $\pm$ 5 %	42,210 $\pm$ 5 %
1 A (280) = (mg/ml)	1.64	1.17
Isoelectric Point	6.61	5.75
Charge at pH = 7	-1.83	-8.44

*The SdaA (PA2443) and SdaB (PA5379) protein sequences were obtained from Pseudomonas.com and analyzed using Protean LaserGene DNASTar 2004.

Table 3. Comparison of *P. aeruginosa* SdaA and SdaB proteins to its homologs

			Percent Similarity								
			1	2	3	4	5	6	7	8	9
			<i>E. coli</i>	<i>P. aeruginosa</i>	<i>M. tuberculosis</i>	<i>C. jejuni</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. pyogenes</i>	<i>Y. pestis</i>	<i>M. bovis</i>
			SdaA	SdaA	SdaA	SdaA	SdaB	SdaB	SdaA	SdaA	SdaA
D i v e r g e n c e	1	<i>E. coli</i> SdaA	***	51.3	48.0	42.3	76.9	51.8	9.6	84.1	48
	2	<i>P. aeruginosa</i> SdaA		***	54.4	41.9	51.6	62.0	12.0	51.5	54.4
	3	<i>M. tuberculosis</i> SdaA			***	39.6	47.7	52.6	12.0	47.7	100
	4	<i>C. jejuni</i> SdaA				***	42.5	40.7	12.3	42.5	39.6
	5	<i>E. coli</i> SdaB					***	52.5	12.0	76	47.7
	6	<i>P. aeruginosa</i> SdaB						***	11.1	51.5	52.6
	7	<i>S. pyogenes</i> SdaA							***	10.2	11.7
	8	<i>Y. pestis</i> SdaA								***	47.1
	9	<i>M. bovis</i> SdaA									***

Clustal analysis was done using *P. aeruginosa* SdaA and SdaB, *E. coli* and SdaB, *M. tuberculosis* SdaA, *M. bovis* SdaA, *C. jejuni* SdaA, *Y. pestis* SdaA and *S. pyogenes* SdaA.

Table 4. Colony size and percentage survival of PAO1 and its isogenic *sda* mutants on various media.*

Media^a	PAO1		PAO<i>sdaA</i>		PAO<i>sdaB</i>	
	Size (cm)	%	Size (cm)	%	Size (cm)	%
Glucose	4	100	4.5	100	4	100
Ser-Gly-Leu	3.5	89	2	85	3	61
Ser-Gly	3.5	88	1.5	73	3	81
Ser-Leu	1	63	0.8	78	1	48
Serine	1	78	0.5	79	1	71
Glycine	2.5	87	1.3	80	2.5	64
Leucine	3	63	1	74	4	50
NIV	1	91	0.5	87	1	62
NMM	0	0	0	0	0	0

*Strains PAO1, PAO*sdaA*, and PAO*sdaB* were plated on differential media. Colony size and percentage colony survival was recorded after 3 days of growth. NIV – Newman Isoleucine-Valine Media; NMM – Newman minimal media

Table 5. Doubling Times of PAO1 and L-SD Mutants.

Strains ^a	Doubling Times ^b (min)			
	LB	NIV	NIV	NIV
		Glu	Ser-Gly-Leu	Ser-Gly
PAO1	37.4	80.5	210.2	285
PAOsdaA	38.5	82	245.3	811.9
PAOsdaB	37.8	80.9	265	681.8

a. The L-serine deaminase mutants, PAOsdaA and PAOsdaB, are isogenic with PAO1 containing (ISphoA/hah) insertions at the sdaA and sdaB loci respectively.

b. *P. aeruginosa* was grown overnight in glucose minimal media and subcultured in minimal media with serine and amino acid supplements. The OD600 was recorded every hour for 16 hours and the doubling time calculated.

Table 6. L-serine deaminase activity of *sda* mutants.

Strains ^a	L-Serine Deaminase Activity ^b			
	Ser-Gly-Leu	Glu	Glu-Gly	Glu-Leu
PAO1	6.8	2.1	2.4	1.5
PAO<i>sdaA</i>	5.2	1.8	1.9	1
PAO<i>sdaB</i>	2.7	1.7	2.4	0.8

a. The L-serine deaminase mutants, PAO*sdaA* and PAO*sdaB*, are isogenic with PAO1 containing insertions of (ISphoA/hah) in each loci.

b. L-Serine-Deaminase activity was measured after 14 hr growth on NIV media with serine, glycine and leucine (Ser-Gly-Leu), with glucose (Glu), glucose and glycine (Glu-Gly) and glucose and lysine (Glu-Leu). The activity is expressed as μ g of pyruvate produced

Table 7. L-serine deaminase activity of serine growth (ser) mutants.

Strains ^a	OD ₆₀₀ ^b	L-Serine Deaminase Activity ^c	
		Ser-Gly-Leu	NIV
PAO1	0.014	6.8	N/A
PAOsdA	0.058	5.2	N/A
PAOsdB	0.02	2.7	N/A
Ser1	0.501	5.9	1.2
Ser2	0.323	6.1	1.7
Ser3	0.176	8	N/A
Ser4	0.744	6.3	1.7
Ser5	0.36	7.9	1.6

a. The serine growth mutants, *ser1* to *ser 5*, that can utilize serine, were isolated by continuous restreaking of strain PAO1 on minimal media plates with serine as the sole carbon source.

b. Strains were grown for 36 hr on NIV media with serine as the sole carbon source at which point the OD₆₀₀ value was recorded.

c. L-Serine deaminase activity was measured after 14 hr growth on NIV alone and NIV media with serine, glycine and leucine (Ser-Gly-Leu). The activity is expressed as μ g of pyruvate produced after 50 minute incubation as described in Materials and Methods.

Figure 1. L-Serine catabolism. L-serine is converted to pyruvate via a one step reaction catalyzed by L-serine deaminase (L-SD). One molecule of H₂O is absorbed and one molecule of NH₃ is released during the process. Pyruvate then enters the citric acid cycle yielding one GTP, four NADH₂, and one FADH₂ molecule.

Figure 2. Comparison of L-serine deamination with glycolysis. In comparison to glycolysis which involves ten enzymes ultimately leading to the production of 38 ATPs, L-serine catabolism only requires one enzyme, L-serine deaminase (L-SD) ultimately leading to the production of 15 ATPs. There is a 15:1 ATP to enzyme ratio for L-serine catabolism as compared to a 3.8:1 ATP to enzyme ratio in glycolysis.

Figure 3. Construction of a suicide vector containing insertional inactivated *sdaA* with *aacCI*. The *sdaA* gene was PCR amplified from the PAO1 chromosome and annealed to pCR2.1 forming pCSMLA. The *SmaI* fragment from pUCGM containing *aacCI* was cloned into a unique *SmaI* site on the *sdaA* gene on pCSMLA forming pCSMLA2. A 3,117 bp *HindIII*-*XbaI*-Klenow treated fragment containing the *sdaA::aacCI* fragment was subcloned into a *SmaI* site on the suicide vector pEX100T that contains *sacB* gene for counter selection and named pXSMLA.

Figure 4. Construction of a suicide vector containing insertional inactivated *sdaB* with *aphI*. The *sdaB* gene was PCR amplified from the PAO1 chromosome and annealed to the vector pCR2.1 forming pCSMLB. An *EcoRI* fragment containing the *sdaB* gene from pCSMLB was subcloned into at the *EcoRI* site on pUC19 forming pUSMLB. The 979-bp *StuI* digested *aphI* PCR amplicon from EZ-KM transposon was ligated to *StuI* digested pUSMLB forming pUSMLBK. The 3010-bp *EcoRI*-Klenow treated *sdaB::aphI* from pUSMLBK was subcloned into the *SmaI* site of the suicide vector pEX100T that contains *sacB* gene for counter selection forming pXSMLBK.

Figure 5. Construction of a suicide vector containing insertionally inactivated *sdaB* with *aacCI*. The 855-bp *SmaI* fragment containing *aacCI* cassette from pUCGM was ligated to *StuI* digested pUSMLB (see Figure 6) forming pUSMLBG. The *EcoRI*- Klenow treated *sdaB::aacCI* from pUSMLBG was subcloned into the *SmaI* site of pEX100T forming pXSMLBG.

Figure 6. Construction of complementing plasmids. In order to obtain plasmids harboring *sdaA* and *sdaB* that are efficiently maintained for phenotypic complementation in *P. aeruginosa*, the broad host-range plasmid pME6030 was used. *HindIII*-cut pME6030 was ligated a 2250-bp *HindIII* fragment harboring *sdaA* from pCSMLAH3 forming pMEsdaA. *EcoRI*-cut pME6030 was ligated to a 2049-bp *EcoRI* fragment harboring *sdaB* from pUSMLB (Figure 6) generating pMEsdaB.

Figure 7. Verification of *sdaA* plasmids and complementing plasmids constructed using 1.0 % agarose electrophoresis . Lanes 1, DNA Ladder N3232S (New England Biolabs); **Panel A)** 2. *sdaA* gene amplified from the PAO1 chromosome using the primers PAO*sdaAH3F* and PAO*sdaAH3R* (2346 bp); 3. *Hind*III- cut pCSMLAH3 yielding three fragments of 67, 2250, and 3882 bp; 4. 855-bp *aacI* from pUCGm; 5. *Sma*I-cut pCSMLA (6199 bp); 6. *Hind* III-*Xba*I-cut pCSMLA2 yielding two 3211 and 3819 bp fragments; 7. 3211 bp *sdaA:aacI* from pCSMLA2; 8. *Sma*I- cut pEX100T (5846 bp); 9. *Fsp*I-cut pXSMLA yielding two confirmatory linear DNA 4183 bp and 4874 bp fragments. **Panel B)** 2. 8021 bp *Eco*RI- cut pME6030; 3. 8021 bp *Hind*III-cut pME6030; 4. 2049-bp *Eco*RI fragment with *sdaB* from pUSMLB; 5. 2250 bp *Hind*III fragment with *sdaA* from pCSMLAH3; 6. *Eco*RI-cut pME*sdaB* yielding two fragments of 8021 bp and 2049 bp; 7, *Hind*III-cut pME*sdaA* yielding two fragments of 8021 bp and 2250 bp.

Figure 8. Verification of *sdaB* plasmids constructed using 1.0 % agarose electrophoresis. Lanes 1. DNA Ladder N3232S (New England Biolabs); **2.** *sdaB* gene amplified from the PAO1 chromosome using the primers PAO*sdaB*F and PAO*sdaB*R (2043 bp); **3.** *Eco*RI-cut pCSMLB yielding two fragments of 2049 and 3913 bp; **4.** 2049-bp *Eco*RI from pCSMLB with *sdaB*; **5.** *Eco*RI-cut pUSMLB, yielding the expected 2049 and 2686 bp fragments; **6.** 2049-bp *Eco*RI fragment harboring *sdaB* from pUSMLB; **7.** 979-bp amplicon from EZ-transposon kit harboring *aphI*; **8.** 855-bp *aacCI* amplicon from pUCGm, **9.** *Stu*I- cut pUSMLB (4735 bp); **10.** *Eco*RI-cut pUSMLBK yielding two fragments of 2686 bp and 3010 bp; **11.** *Eco*RI-cut pUSMLBG yielding two fragments of 2686 and 2904 bp; **12.** 3010-bp fragment from pUSMLBK with *sdaB::aphI*; **13.** *sdaB::aacCI* from pUSMLBG; **14.** *Fsp*I-cut pXSMLBK yielding four fragments of 363, 1023, 1714, and 2596 bp; and **15.** *Fsp*I-cut pXSMLBG yielding four fragments of 363, 1023, 1608, and 2596 bp.

Figure 9. Chromosomal location of *P. aeruginosa* *sda* genes. (A). The *sdaA* (PA2443) gene is found at position 2742353 to 2740977 within the *P. aeruginosa* Strain PAO1 chromosome (<http://v2.pseudomonas.com/getAnnotation.do?locusID=PA2443>). **(B).** *P. aeruginosa* *SdaB* is encoded by *sdaB* (PA5379) found at position 6056877 to 6058253 within the PAO1 chromosome (<http://v2.pseudomonas.com/getAnnotation.do?locusID=PA5379>).

A

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1  MSLSVFDLFGIGIPSSSHTVGPMRAAARFAEGLRREGLLEATASIKVELYGSLGATGKG
61  HGSDKAVLLGLEGEQPDVTDTAAIPARLDAIRSSGELRLLGERPIRFVEKEHLALIRKPL
121 AYHPNGMIFRAFDAAGLQVRSREYYSVGGGFVVDDEAAGLDRIVEDRTPLAFPFKTARQL
181 LDHCAREGLSISQLMAENEKAWRPAEETRTGLLRINQVMQDCVEAGCRNEGIMPGGLKVR
241 RRAAALHRQLCQRPEAGLRDALSVLDWVNLYALAVNEENASGGRVVTAPTNGAAGIIPAV
301 LHYYARFIPGADDDGVVRFLLTAAAIGILYKENASISGAEVGCQGEVGVACSMAAGALCE
361 VLGGSVQQVENAAEIGMEHNLGLTCDPVGGLVQVPCIERNAMASVKAINAARMALRGDGQ
421 HFISLDKVIRTMRTGADMKSKYKETARGGLAVNIIEC

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B

Figure 10. SdaA protein analysis. The amino acid sequence of SdaA **(A)** was analyzed via a Kyte Doolittle Hydrophobicity plot **(B)** ran by Colorado State University <http://www.vivo.colostate.edu/molkit/hydropathy/index.htm>.

Figure 11. SdaB protein analysis. The amino acid sequence of SdaB (A) was analyzed via a Kyte Doolittle Hydrophobicity plot (B) ran by Colorado State University <http://www.vivo.colostate.edu/molkit/hydropathy/index.htm>.

Figure 13. *P. aeruginosa* PAO1 growth in various media. PAO1 growth was optimal in rich LB media (●), NIV media with Glucose (■), serine (▲), serine and glycine (×), serine and leucine (◆), serine, glycine and leucine (✕). The growth OD600 was determined for every hour for 15 hours.

Figure 14. Growth characteristics of *P. aeruginosa* *sda* mutants. The growth pattern of PAOsdaA (■) and PAOsdaB (▲) were compared with their isogenic parent strain PAO1 (◆). The cells were cultured in **(A)** LB, **(B)** glucose, **(C)** NIV minimal media with serine, **(D)** NIV minimal media with serine and leucine, **(E)** NIV minimal media serine and glycine, and **(F)** NIV minimal media with serine, glycine and leucine.

Figure 15. The L-serine deaminase enzyme activity of *P. aeruginosa* PAO1. The L-SD activity was measured after culturing the bacteria in NIV media with **(A)** glucose and glycine (NIV Glu-Gly) and, **(B)** serine, glycine and leucine (NIV Ser-Gly-Leu).