Title:

D,D-muramyl endopeptidase Spr contributes to intrinsic vancomycin resistance in *Salmonella enterica* serovar Typhimurium

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ABSTRACT

Gram-negative bacteria are intrinsically resistant to vancomycin, a clinically important antibiotic that targets cell wall synthesis, used in the treatment of Gram-positive bacterial infections. The relative impermeability of the Gram-negative bacterial outer membrane, a feature lacking in Gram-positive bacteria, is believed to play a major role in intrinsic antibiotic resistance. Here we show that deletion of *spr*, a gene encoding a periplasmic D,D-muramyl endopeptidase involved in cell wall peptidoglycan remodeling, sensitizes the Gram-negative bacterium *Salmonella enterica* serovar Typhimurium to vancomycin by a mechanism associated with increased bacterial autolysis. A screen for suppressor mutants revealed that deletion of *tsp*, encoding a periplasmic carboxypeptidase involved in processing Spr and PBP3, restored resistance to vancomycin and reversed the autolysis phenotype. Interestingly, deletion of *tsp* alone sensitized *S.* Typhimurium to the anti-staphylococcal antibiotic novobiocin, a phenotype that was reversed by the additional deletion of *spr*. We discuss how the interplay between the activities of Spr and Tsp could explain differential sensitization to Gram-positive-specific antibiotics. In conclusion, the data imply that the activity of the periplasmic D,D-muramyl endopeptidase Spr contributes to intrinsic vancomycin resistance in *S.* Typhimurium. This suggests that developing compounds that target specific cell wall peptidases might be a path to expanding the activity spectrum of traditional Gram-positive antibiotics.

52

53 **IMPORTANCE**

54 Vancomycin is an important antibiotic that targets cell wall synthesis and is used to
55 treat Gram-positive bacterial infections. The intrinsic resistance of Gram-negative
56 bacteria to vancomycin is believed to be due to the presence of an outer membrane
57 that is relatively impermeable, preventing vancomycin from reaching its
58 periplasmic cell wall target. Here we show that inactivation of a periplasmic enzyme
59 called Spr, involved in peptidoglycan remodelling, sensitizes the Gram-negative
60 bacterium *Salmonella enterica* serovar Typhimurium to vancomycin, and does so
61 without increasing outer membrane permeability. Sensitization is associated with
62 increased autolysis and can be suppressed by inactivation of Tsp, an enzyme
63 involved in processing Spr and PBP3. This discovery suggests that developing
64 compounds that target specific cell wall peptidases, such as Spr, might be a path to
65 expanding the activity spectrum of traditional Gram-positive antibiotics.

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70 **INTRODUCTION**

71 Gram-negative bacteria are characterized by having two membranes, the inner
72 membrane and outer membrane. The outer membrane of enterica bacteria, due to
73 its relative impermeability, provides an intrinsic resistance mechanism against
74 many antibiotics, including erythromycin (1,2), novobiocin (3), rifampicin (1), and
75 vancomycin (4,5), limiting their clinical usefulness to Gram-positive species.

76

77 The bacterial cell wall, located between these two membranes, is composed of
78 peptidoglycan (murein) and its synthesis is the target of clinically important classes
79 of antibiotics, such as the β-lactams and the glycopeptides (6). Peptidoglycan is a
80 composite mesh consisting of repeated subunits of N-actetyl-β-D-glucosamine
81 (GlcNAc) and N-acetyl-β-D-muramic acid (MurNAc) disaccharide, cross-linked by
82 peptide bridges (7). As such, peptidoglycan contributes to bacterial shape, septum
83 formation at the point of cell division, and maintenance of the internal turgor
84 pressure of the bacterial cell.

85

86 As the bacterial cell grows and divides the peptidoglycan network has to grow to
87 accommodate this expansion. For this, peptidoglycan needs to change its size and
88 shape. To enable these dynamics, various amidases, endopeptidases and
89 glycosylases act together with so-called penicillin-binding proteins (PBPs) to shape
90 the peptidoglycan (8). While the PBPs have the major responsibility of catalyzing
91 the formation of interpeptide bridges between overlapping GlcNAc-MurNAc
92 polymers to build the peptidoglycan mesh, the murein endopeptidases are tasked

93 with cleaving interpeptide bridges to facilitate the incorporation of new GlcNAC-
94 MurNAc polymers into the growing peptidoglycan mesh. The importance of
95 correctly balancing these opposing activities is illustrated by the fact that blocking
96 PBP activity in *E. coli* results in bacterial autolysis (9).
97
98 The increasing frequency of clinical isolates producing extended-spectrum β-
99 lactamases (ESBL) is limiting the effectiveness of antibiotics that target cell wall
100 synthesis amongst Gram-negative species (10). The search for new antibiotics to
101 treat Gram-negative bacterial infections would be advanced by a better
102 understanding of bacterial cell wall homeostasis at the level of peptidoglycan.
103 Because it is genetically amenable, *E. coli* has been the main focus for studies on the
104 activities of peptidoglycan-modulating enzymes. From these studies a consensus
105 view has emerged that, apart from PBP3 (11), each of the glycolytic and endopeptic
106 hydrolases are individually dispensable for bacterial viability. Accordingly,
107 inactivation of any one (or sometimes more than one) of the genes encoding the
108 enzymes (PBP4: (12–14), PBP5: (12,15–17), PBP6: (12,18), PBP6b: (19),
109 PBP7/PBP8: (12,20), reviewed in: (21)) does not prevent bacterial growth under
110 laboratory conditions. While this might imply a high degree of functional
111 redundancy, it does not exclude the possibility that some or all of these enzymes
112 may have unique functions under specific *in vivo* conditions.
113
114 A recent study (22) confirmed D,D-muramyl endopeptidase activity for three
115 additional *E. coli* proteins; Spr, YebA and YdhO (renamed in *E. coli* to MepS, MepM

116 and MepH (23)). More specifically the study presented data that Spr and YebA
117 might be endopeptidases with unique functions in that they were unable to
118 construct an Δ*spr*Δ*yebA* mutant in *E. coli* (22).
119
120 *S.almonella enterica* serovar Typhimurium (referred to throughout the text as *S.*
121 Typhimurium) is a Gram-negative enterobacterium related to *E. coli* and *Shigellae.*
122 *S*. Typhimurium encodes proteins with high sequence similarity to the Spr, YebA,
123 and YdhO proteins of *E. coli* and *Shigella flexneri* (Fig. S1). Interestingly, while YdhO
124 of *E. coli* and *S. flexneri* are highly similar throughout their entire length (90%
125 similarity), YdhO of *S.* Typhimurium, diverges strongly from the others in the N-
126 terminal half of the protein (68% similarity for the entire length when compared to
127 *E. coli*) (Fig. S1). Considering the potential importance of Spr and YebA for viability
128 of *E. coli* (22), and the structural divergence of YdhO in *S*. Typhimurium compared
129 to the other enteric bacteria, we studied the phenotypes of *S.* Typhimurium mutants
130 in which these genes were deleted, either singly or in combination. Here we report
131 the construction and characterization of Δ*spr,* Δ*yebA* and Δ*ydhO* mutants in terms of
132 their growth and antibiotic susceptibility phenotypes. We report that the intrinsic
133 vancomycin resistance of *S.* Typhimurium requires Spr activity, but only in a Tsp
134 (Prc) protease-proficient background.
135

136 **RESULTS**

137 **Construction of Δ*spr*, Δ*yebA* and Δ*ydhO* mutants**. To assess the functional
138 similarity of the *E. coli* MepS, MepM and MepH proteins and their *S*. Typhimurium
139 homologues (Spr, YebA and YdhO), we constructed single and double deletion
140 mutants of *spr*, *yebA* and *ydhO* in *S*. Typhimurium SR-11 (for details, see Materials
141 and Methods). In agreement with previous data from *E. coli* (22) we were not
142 successful in creating a Δ*spr*Δ*yebA*Δ*ydhO* triple mutant.

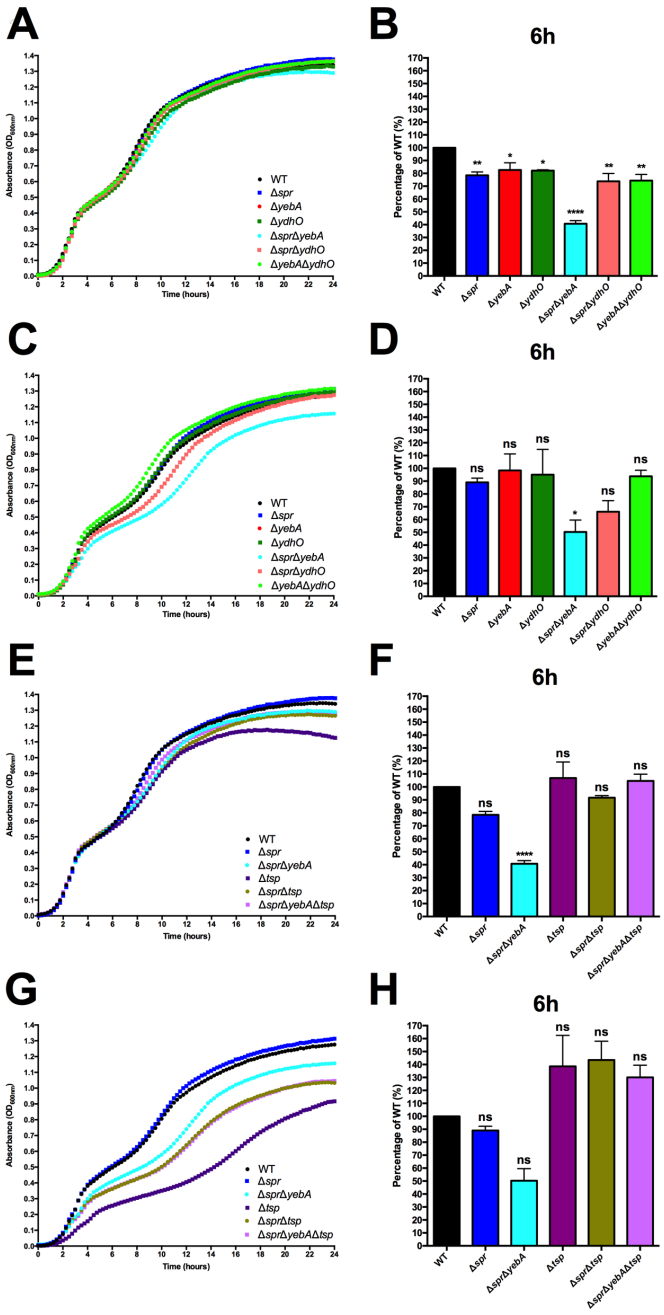
143

144 **Growth characteristics of mutants**. The exponential growth rates of the *S*.
145 Typhimurium mutants in LB and TY broth were compared to that of wild-type *S.*
146 Typhimurium SR-11. In agreement with observations made in *E. coli* (22) none of
147 the single or double deletion mutants revealed any significant difference in the
148 overall shape of their growth curves (Fig. 1A and 1C). All mutants had a similar
149 logarithmic growth rate, and reached a similar optical density in stationary phase.
150 This also applied to the Δ*spr*Δ*yebA* mutant, a construct of which cannot be
151 maintained in *E. coli* without the simultaneous ectopic expression of *spr* (21).

152

153 To examine colony-forming ability, samples were taken in early log phase (2.5
154 hours), mid-log phase (6 hours) and stationary phase (11 hours) after inoculation
155 of the broth cultures. The absorbance at OD_{600nm} was normalised for each of the
156 samples after which viable counts of the samples were determined on LB agar
157 plates. Apart from the Δ*spr* mutants all strains yielded CFU comparable to that of
158 the wild-type at mid-log phase. All of the Δ*spr* mutants had a small reduction in CFU,

159 while for the Δ*spr*Δ*yebA* mutant there was a much greater reduction in CFU in
160 relation to the normalised OD_{600nm} (Fig. 1B and 1D). We observed similar CFU
161 results for early log phase and stationary phase (Fig. S2B and S2D).
162
163 Taken together, these findings suggest a high degree of redundancy regarding the
164 Spr, and YebA endopeptidases in *S*. Typhimurium. However, the observation that
165 the Δ*spr* mutants were each associated with a reduced CFU yield from broth
166 cultures hinted at a role for Spr in colony formation, which is amplified in a
167 Δ*spr*Δ*yebA* mutant.



168

169 **Figure 1. Growth characteristics of S. Typhimurium strains**. Bacterial strains were diluted to an OD_{600nm} of 0.01 from
170 overnight LB broth cultures into LB (A & E) or TY (C & G) broth where after increase in bacterial density was followed for 24
171 hours. Viable counts were recovered from LB (B & F) and TY (D & H) broth from mid-log phase (6 hours after inoculation).
172 Culture samples were first diluted to an equal OD_{600nm} value, where after viable counts were determined and normalised to
173 that of the wild-type. The graph shows the mean value and the standard error of the mean. One-way ANOVA with Dunnett's
174 correction was used to analyze the statistical significance of the mutants compared to the wild-type (p-values; ns = $p > 0.05$, *
175 = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$).

176 **Antibiotic and detergent sensitivities**. It was previously noted that *E. coli* strains
177 simultaneously defective in several cell wall turnover enzymes can display an outer
178 membrane permeability phenotype, phenotypically observed by an increase in
179 sensitization to many antibiotics (21). Hence, we screened the panel of *S.*
180 Typhimurium mutants for altered sensitization to six different antibiotics using the
181 disc diffusion method. The antibiotics tested were penicillin G, novobiocin,
182 polymyxin B, rifampicin, tetracycline and vancomycin. As comparator strains we
183 used wild-type *S*. Typhimurium SR-11, and three isogenic LPS mutants known to
184 have an outer membrane permeability defect (24). Sensitivity testing was
185 conducted using low osmolarity TY medium. The rationale for this was that high
186 salt concentrations reduce the sensitivity of *E. coli* to selected antibiotics (25), and
187 that low osmolarity medium would favour expression of the more permeable outer
188 membrane porin OmpF (26,27), thus increasing the probability of detecting any
189 sensitization.
190
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Antibiotic (⊘ mm)	Vancomycin	Novobiocin	Polymyxin B	Tetracycline	Rifampicin	Penicillin G
WT	0 (100)	0	13	18	16	27 (6.25)
Δ*spr*	13 (12.5)	0	12	17	17	27 (6.25)
Δ*yebA*	0 (100)	0	13	17	17	28
Δ*ydhO*	0 (100)	0	13	16	15	26
Δ*spr*Δ*yebA*	25 (3.125)	10	15	20	40	38
Δ*spr*Δ*ydhO*	14 (25)	0	12	16	20	28
Δ*yebA*Δ*ydhO*	0 (100)	0	13	16	18	27
Δ*tsp*	0 (100)	15	14	21	18	34
Δ*spr*Δ*tsp*	0 (100)	0	13	19	16	26
Δ*spr*Δ*yebA*Δtsp	0 (100)	0	13	20	17	27
Δ*nlpI*::Kan^R	0	12	15	23	16	27
Δ*spr*Δ*nlpI*::Kan^R	12	0	14	20	16	23
Δ*spr*Δ*yebA*Δ*nlpI*::Kan^R	17	8	14	20	19	25
Δ*mepA*::Kan^R	0	0	14	18	15	25
Δ*nlpC*::Kan^R	0	0	14	19	15	24
Δ*pbpG*::Kan^R	0	0	14	20	14	23
Δ*rfaC*::Chl^R	25	38	42	28	35	40
Δ*rfaG*::Kan^R	17	27	35	21	28	22
Δ*rfaP*::Chl^R	15	29	31	20	26	18

Table 1. Antibiotic sensitivity profiles. Inhibition zones in millimeters (mm) on TY agar when applied round 6 mm Whatman 3 filter paper bits containing selected antibiotics. Numbers in parenthesis within the table are the minimum inhibitory concentrations in μg/ml as determined from broth dilutions of the antibiotics.

As expected, the three control LPS mutants, Δ*rfaC* (*waaC*), Δ*rfaG* (*waaG*), and Δ*rfaP* (*waaP*) were each sensitized to polymyxin B, novobiocin, rifampicin and vancomycin compared to the wild-type (Table 1). The Δ*spr* mutant was strongly sensitized to vancomycin (inhibition zone increased from 0 to 13mm) but not to any of the other antibiotics tested. The vancomycin sensitization associated with *spr* deletion was confirmed using a drop-on-lawn assay and MIC determinations (Table 1, Fig. 2A). Subsequent MIC determinations demonstrated that the intrinsic vancomycin resistance of *Salmonella* was reduced 8-fold for the Δ*spr* mutant and 32-fold for the Δ*spr*Δ*yebA* mutant, while the Δ*yebA* mutant did not show vancomycin sensitization (Table 1). Also, the Δ*spr*Δ*yebA* mutant revealed a moderate increase in sensitization to penicillin G and rifampicin while the Δ*yebA* mutant did not reveal any increase in sensitization (Table 1).

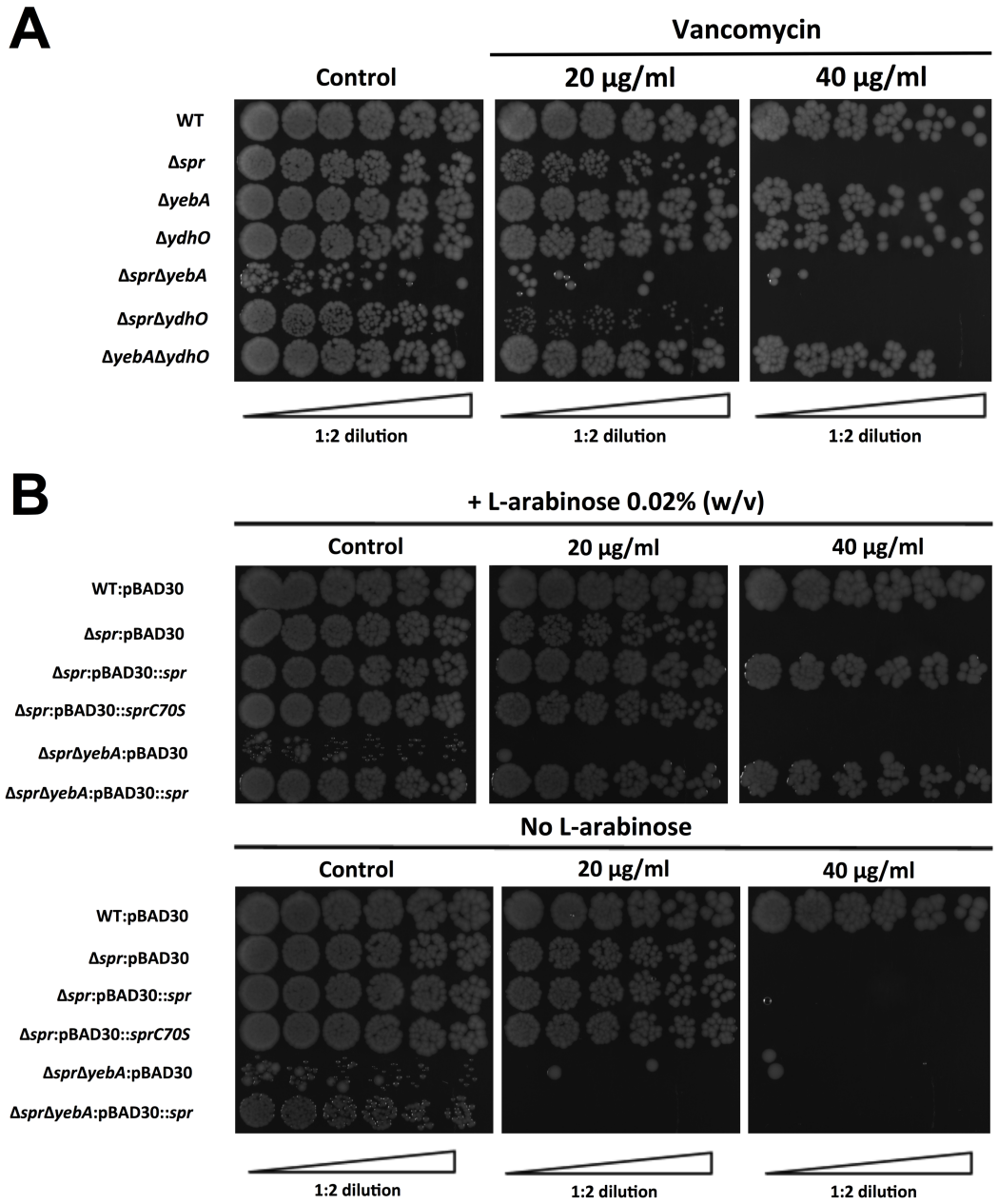
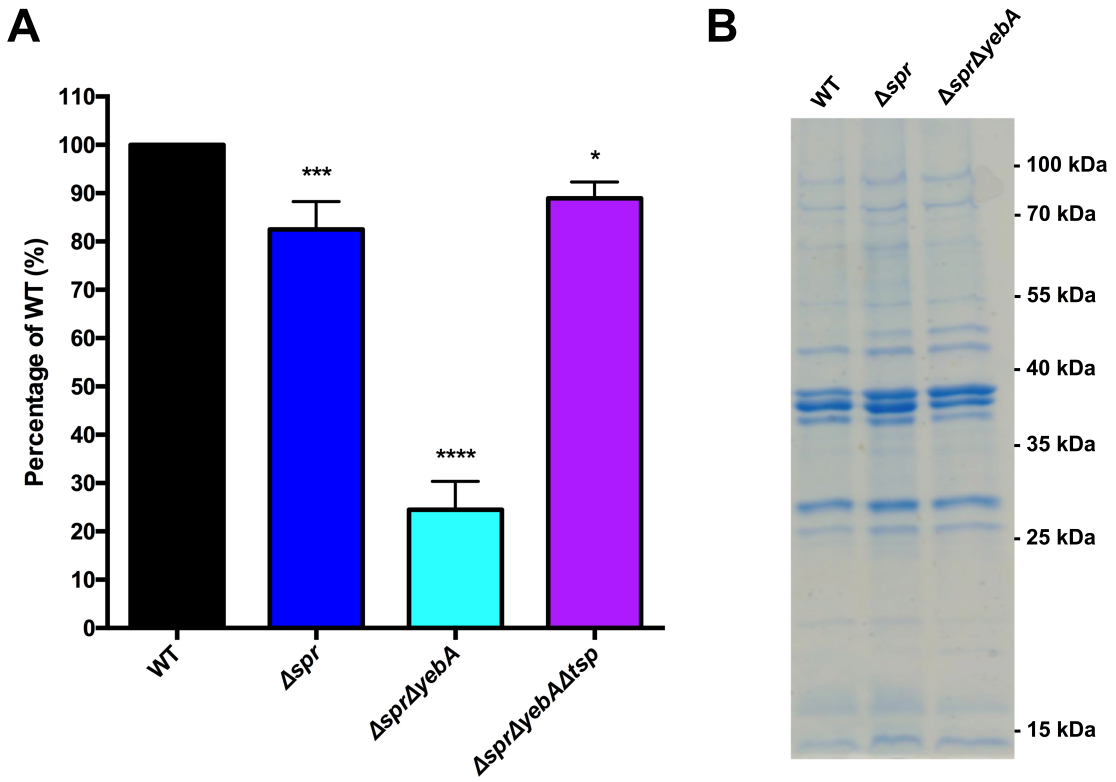


Figure 2. Drop-on-lawn visualization of vancomycin sensitivity in D,D-muramyl endopeptidase mutants. (A) Wild-type and each single and double mutants of the D,D-muramyl endopeptidases specified were 1:2 serially diluted and 5µl of each dilution was spotted on either TY agar plates as control, or TY agar plates supplemented with 20 µg/ml or 40 µg/ml of vancomycin. (B) Wild-type, Δ*spr* and Δ*spr*Δ*yebA* mutants harboring either vector control pBAD30, pBAD30::*spr* for full complementation or pBAD30::*sprC70S* as catalytic knockout were serially diluted and spread on TY agar plates, or TY agar plates supplemented with 20 µg/ml or 40 µg/ml of vancomycin, either containing L-arabinose or not.

225 Next we introduced the Δ*spr* mutation into *S.* Typhimurium SL1344 and 14028s and
226 assayed for vancomycin sensitivity by MIC determination. The MIC for the wild-
227 type and Δ*spr* mutant in both *S*. Typhimurium SL1344 and 14028s was identical to
228 the MIC previsouly measured for the wild-type and Δ*spr* mutant of *S.* Typhimurium
229 SR-11 (100μg/ml and 12.5 μg/ml respectively), thus showing that the vancomycin
230 sensitivity of the Δ*spr* deletion is not restricted to the SR-11 background.
231
232 In *E. coli* overproduction of PBP7 suppresses thermosensitive growth associated
233 with a *mepS* (*spr*) mutation (28), suggesting that PBP7 and MepS may function in
234 parallel pathways. We deleted *pbpG*, coding for the PBP7 homologue in *S*.
235 Typhimurium yet the Δ*pbpG* mutant was not sensitized to any of the antibiotics in
236 the test panel (Table 1). We also created deletion mutants for the *mepA* and *nlpC*
237 homologues in *S*. Typhimurium, each coding for endopeptidases related to Spr (8).
238 Neither of the two mutations resulted in any increased sensitization to the
239 antibiotics in the test panel (Table 1).
240
241 To further evaluate potential cell wall defects in the Δ*spr* and Δ*spr*Δ*yebA* mutants,
242 we tested their sensitivity to a detergent by incubating them in 0.5% sodium
243 deoxycholate (DOC). In this assay the Δ*spr*Δ*yebA* mutant revealed a clear
244 sensitization to DOC exposure (Fig. 3A).
245
246 The outer membrane protein profile of a Gram-negative bacteria can affect its
247 antibiotic sensitivity (29). Thus we compared outer membrane protein profiles of

248 the wild-type, Δ*spr* and Δ*spr*Δ*yebA* mutants on SDS-PAGE gels. We did not observe
249 any significant difference in outer membrane protein profile for the mutants as
250 compared to the wild-type (Fig. 3B), arguing that the increased vancomycin
251 sensitization is not caused by a major outer membrane disruption.
252



253
254 **Figure 3. Detergent sensitivity and membrane protein profile of D,D-muramyl endopeptidase mutants**. (A) For
255 probing detergent sensitivity 10^5 bacteria of each strains was incubated in 0.5% DOC for 30 min, where after the bacterial
256 viable colonies were enumerated. Results are presented as relative CFU yield in relation to the wild-type. The graph shows the
257 mean value and the standard error of the mean of at least three independant replicates. (B) SDS-PAGE gel revealing the outer
258 membrane protein profiles of the wild-type and mutant strains. The positions of pre-stained molecular weight markers are
259 indicated at the right. The standard used is PageRuler Prestained Protein Ladder (#26616, Thermo Scientific).
260
261

262 We conclude that the lack of Spr, but not the endopeptidases YebA, YdhO, PBP7,
263 MepA or NlpC, results in sensitization to vancomycin in an otherwise intrinsically
264 resistant *S.* Typhimurium. Sensitization to vancomycin does not appear to reflect a
265 general outer membrane permeabilization in the Δ*spr* mutant, while the additional
266 increase in sensitization of the Δ*spr*Δ*yebA* mutant might be a in part a consequence
267 of an additional outer membrane destabilisation as evidenced by the sensitization
268 to detergent, novobiocin and rifampicin.
269
270 **Genetic complementation of Δ*spr* mutants**. We used genetic complementation to
271 confirm the contribution of *spr* to the intrinsic vancomycin resistance of *S*.
272 Typhimurium. We cloned the *spr* gene from *S*. Typhimurium SR-11 under the
273 control of the arabinose-inducible promoter in the vector pBAD30. We also
274 generated a point mutation in this plasmid replacing the codon for the conserved
275 catalytic Spr cysteine (Fig. S1) with a codon for serine, creating a C70S mutation in
276 the mature protein (22,30). Introducing the cloned native *spr* gene into either the
277 Δ*spr* or Δ*spr*Δ*yebA* mutant fully restored vancomycin resistance, whereas the
278 plasmid coding for a catalytically inactive Spr variant did not do so (Fig. 2B). We
279 concluded that Spr is required for the intrinsic vancomycin resistance of *S.*
280 typhimurium and that this phenotype is dependent on the expression of a
281 catalytically active Spr.
282
283 **The Δ*spr* mutation promotes autolysis**. When counting CFU from liquid cultures
284 of the Δ*spr*Δ*yebA* mutant on LB agar plates we noted that the colonies of the

285 Δ*spr*Δ*yebA* mutant were visibly smaller in size compared to wild-type (Fig. 4A). This
286 led us to assess the number of viable bacteria in the colonies. We suspended
287 individual colonies of wild-type, Δ*spr* and Δ*spr*Δ*yebA* mutants in PBS, normalized
288 the OD_{600nm} and counted CFU from each suspension. In doing this, we noted a small
289 reduction in CFU for the Δ*spr* mutant, and substantial reduction in CFU for the
290 Δ*spr*Δ*yebA* mutant (Fig. 4B).
291
292 To further assess the significance of the smaller colonies with regards to bacterial
293 viability we suspended colonies in PBS and analyzed the supernatants for proteins
294 by SDS PAGE and protein staining. The supernatant of the Δ*spr* mutant was similar
295 to that of the wild-type, while the profile of the Δ*spr*Δ*yebA* mutant showed evidence
296 of bacterial lysis (Fig. 4C).

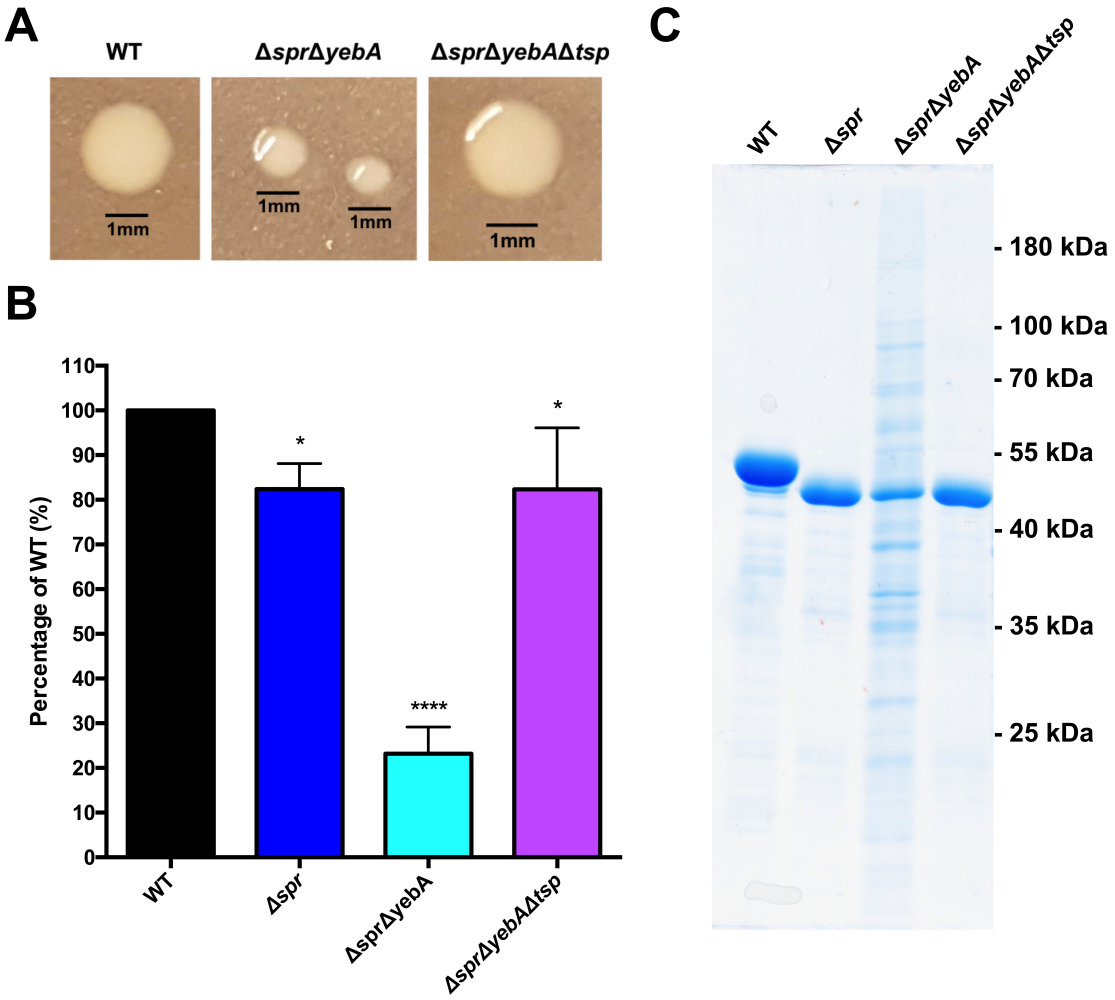


Figure 4. Growth characteristics and autolysis of the Δ*sprΔyebA* mutant on LB agar plate. (A) The size of the bacterial colonies. Bacterial colonies imaged with a Samsung Galaxy 8S smartphone. The shine on the colonies does not reflect mucosity of colonies, only the reflection of light during imaging. (B) Relative viable counts. Colonies were first diluted to an equal OD_{600nm} value, where after viable counts were determined and normalised to that of the wild-type. The graph shows the mean value and the standard error of the mean of at least three independant replicates.. One-way ANOVA with Dunnett's correction was used to analyze the statistical significance of the mutants compared to the wild type (p-values; * = $p < 0.05$, **** = $p < 0.0001$). (C) SDS-PAGE protein gel profiles of corresponding bacteria-free supernatant. The positions of pre-stained molecular weight markers are indicated at the right. The standard used is PageRuler Prestained Protein Ladder (#26616, Thermo Scientific).

309 The indicative bacterial lysis of the Δ*spr*Δ*yebA* mutant (Fig. 4C) suggested that the
310 mutant had an autolytic phenotype. To quantify lysis we introduced a plasmid,
311 pKTH3088 constitutively expressing *E. coli* β–galactosidase, into the wild-type, and
312 the Δ*spr* and Δ*spr*Δ*yebA* mutants of *S*. Typhimurium. β–galactosidase is a
313 cytoplasmic enzyme and would be released into the medium in the event of lysis.
314 This release was measured by the enzymatic activity of the β–galactosidase and
315 quantified as the relative amount of autolysis for the wild-type and mutant strains.
316
317 We first performed β-galactosidase activity assays as a function of increasing
318 concentrations of penicillin G. Both the wild-type and Δ*spr* mutant reached a similar
319 plateau level of β-galactosidase activity (Fig. 5A). However, the kinetics of β-
320 galactosidase release were different, with the Δ*spr* mutant releasing more β-
321 galactosidase at lower concentrations (2 and 4 μg/ml) of penicillin G than the wild-
322 type (Fig. 5A), even though both strains had a similar MIC for penicillin G (Table 1).
323 For the Δ*spr*Δ*yebA* mutant the trend for penicillin G-induced β-galactosidase release
324 was more pronounced when compared to the wild-type, but also reached a similar
325 plateau as the wild-type at higher concentrations of penicillin G (Fig. 5B). We then
326 conducted similar lysis experiment replacing penicillin G with vancomycin. β-
327 galactosidase activity was released substantially more from the Δ*spr* and Δ*spr*Δ*yebA*
328 mutants at lower vancomycin concentrations, reflecting their lower MIC for
329 vancomycin (Fig. 5C and 5D).
330
331

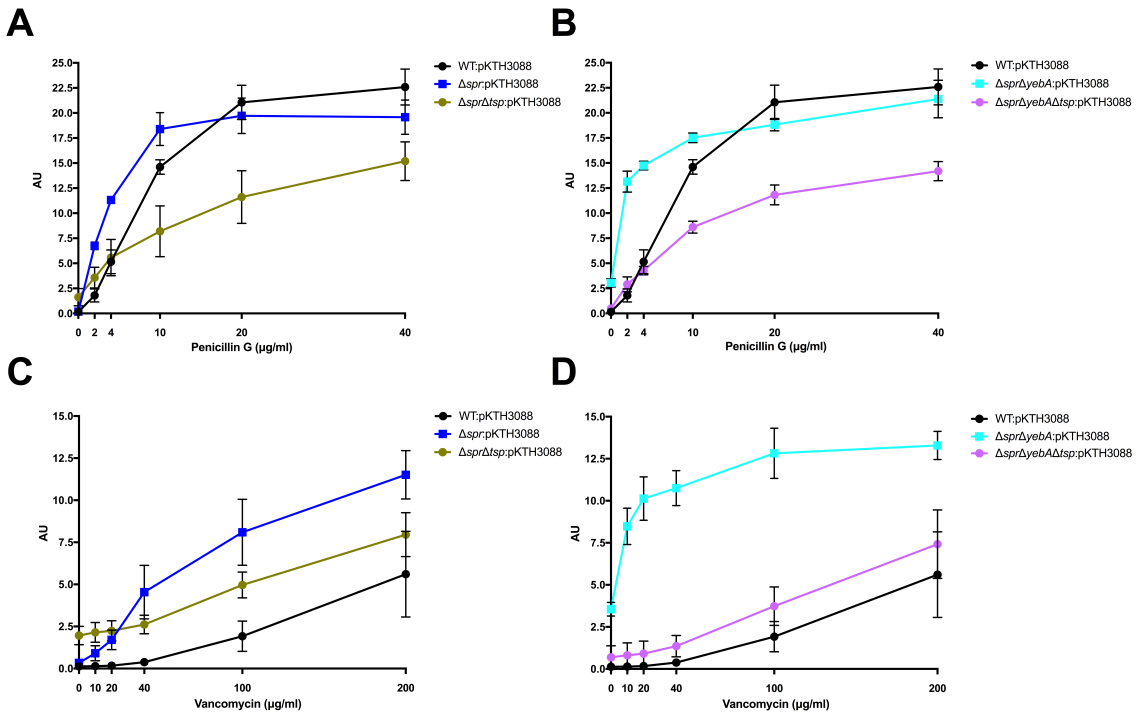


Figure 5. Antibiotic induced autolysis. Release of cytoplasmic β-galactosidase in the presence of increasing concentrations of pencillin G (A & B) or vancomycin (C & D) after 1 hour of incubation at 37°C. Graph presents the mean values and standard error of the mean of at least three independent replicates. The statistics between mutants and wild-type are presented in Figure S4.

In *E. coli*, blocking protein synthesis prevents penicillin-induced autolysis (9,31). We found that the addition of tetracycline to the medium prevented both penicillin G-induced and vancomycin-induced release of β-galactosidase from the wild-type and the Δ*spr* and Δ*spr*Δ*yebA* mutants (Fig. S3). This implied that the release of β-galactosidase observed in the presence of the antibiotics was indeed due to autolysis.

Thus, the Δ*spr* and Δ*spr*Δ*yebA* mutants express an autolytic phenotype that correlates with their sensitivity to vancomycin and is indicative of a mechanistic

347 explanation for the vancomycin sensitization. The greater autolytic propensity of
348 the Δ*spr*Δ*yebA* mutant, when compared to the Δ*spr* mutant, is likely also the
349 explanation for the relatively smaller colony size, reduced CFU yield, and the
350 supernatant protein profile of the Δ*spr*Δ*yebA* mutant (Fig. 4).
351
352 **Isolation of Δ*spr* vancomycin resistant suppressor mutations**. Vancomycin-
353 tolerant mutants were selected in the Δ*spr*Δ*yebA* background using a vancomycin
354 gradient TY agar plate. Twelve independent vancomycin-tolerant mutants were
355 analyzed by whole genome sequencing. Eleven of the twelve mutants carried
356 deletions covering the same region (nucleotides 1,920,000 - 1,965,000) in the *S.*
357 Typhimurium LT2 reference genome (Fig. 6A). At the centre of this region is *tsp*,
358 encoding a periplasmic carboxypeptidase. The remaining suppressor mutant
359 carried a point mutation within *tsp* itself (Fig. 6A). These data suggest that
360 inactivation of *tsp* is the common feature of mutations that suppress the
361 vancomycin sensitivity phenotype of the Δ*spr*Δ*yebA* mutant.
362
363 To confirm the *tsp* mutations as suppressors, we deleted *tsp* in the Δ*spr*Δ*yebA*
364 mutant, as well as the individual genes that mapped upstream and downstream of
365 *tsp* (*proQ* and *htpX*) in the Δ*spr*Δ*yebA* mutant. Out of these constructs only deletion
366 of *tsp* generated a vancomycin resistant phenotype in the Δ*spr*Δ*yebA* mutant (Table
367 1, Fig. 6B). In addition, when the *tsp* deletion was introduced into the Δ*spr* mutant it
368 converted the phenotype from vancomycin-sensitive to vancomycin-resistant
369 (Table 1, Fig. 6B). Conversely, when a cloned *tsp* gene was introduced into the

370 vancomycin resistant Δ*spr*Δ*tsp* and Δ*spr*Δ*yebA*Δ*tsp* mutants the phenotypes of each

371 were converted to vancomycin-sensitive (Fig. 6C).

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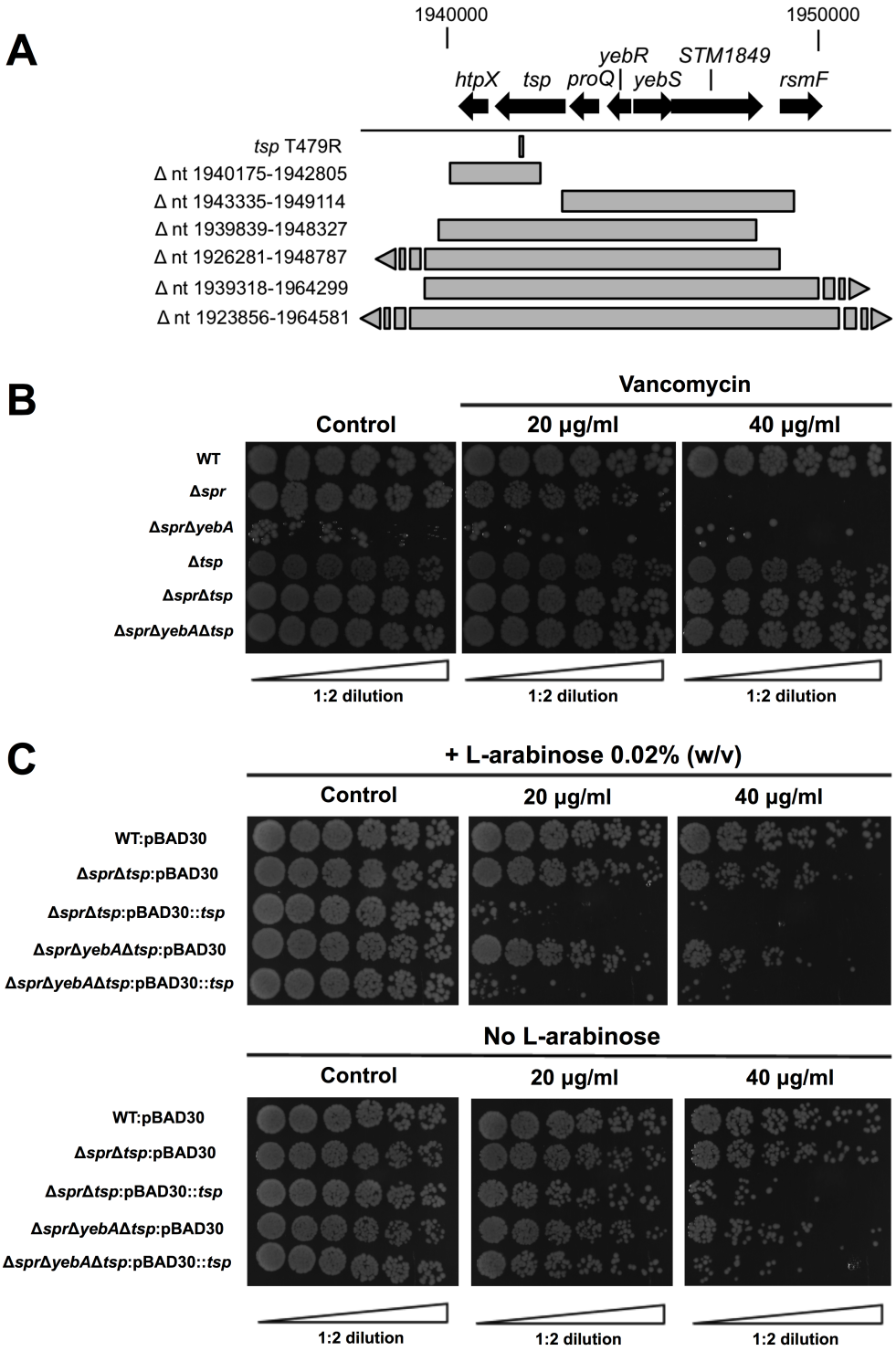

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374 **Figure 6. Suppression of vancomycin sensitivity in Δ*spr* mutant backgrounds.** (A) Genomic visualization of vancomycin
375 tolerant suppressor mutations with the bars representing genomic deletions, except for *tsp* T479R where the bar points to the
376 position of a point mutation. (B) Drop-on-lawn visualization of vancomycin tolerance using a defined Δ*tsp* mutation. Wild-
377 type, Δ*tsp,* Δ*spr* and Δ*spr*Δ*yebA* mutant, and their Δ*tsp* suppressed mutants were 1:2 serially diluted and 5µl of each dilution
378 was spotted on either TY agar plates as control, or TY agar plates supplemented with 20 µg/ml or 40 µg/ml of vancomycin.
379 (C) Wild-type and the Δ*tsp* suppressed mutants harboring either vector control pBAD30 or full complementation pBAD30::*tsp*
380 were serially diluted and spread on TY agar plates, or TY agar plates supplemented with 20 µg/ml or 40 µg/ml of vancomycin,
381 either containing L-arabinose or not.
382

383 The Δ*tsp* mutation also reverted the general antibiotic sensitization of the
384 Δ*spr*Δ*yebA* mutant (Table 1). Furthermore, the smaller colony size, reduced CFU,
385 and supernatant protein profile, were restored to wild-type patterns in a
386 Δ*spr*Δ*yebA*Δ*tsp* mutant (Fig. 4, 1F, 1H, S2H and S2F). In addition, introduction of the
387 *tsp* deletion into the Δ*spr* and Δ*spr*Δ*yebA* mutants suppressed the autolysis by
388 reducing their release of β-galactosidase in the presence of penicillin G or
389 vancomycin (Fig. 5). Thus, a *tsp* deletion acted as a general suppressor mutation for
390 Δ*spr* and Δ*spr*Δ*yebA* mutant phenotypes.
391

392 In *E. coli*, Tsp co-purifies with the outer membrane lipoprotein NlpI, which assists
393 Tsp in degrading MepS (Spr) (23). Furthermore, *nlpI* deletions suppress a
394 temperature-sensitive phenotype of an *E. coli* Δ*mepS* mutant (32) implying a
395 functional connection between Tsp, NlpI and MepS in *E. coli*. We deleted *nlpI* in the
396 *S*. Typhimurium Δ*spr* and Δ*spr*Δ*yebA* mutants to test whether this would affect the
397 antibiotic sensitivity profile of the mutants. In this, the Δ*nlpI*Δ*spr*Δ*yebA* mutant
398 retained vancomycin sensitivity (Table 1).
399

400 **An *spr* deletion acts as a suppressor for Δ*tsp* mutant phenotypes**. In agreement
401 with the published phenotype of an *E. coli tsp* mutant (33), also the *S*. Typhimurium
402 Δ*tsp* mutant was sensitized to novobiocin (Table 1). Additionally, we noted a
403 marked detergent sensitization when incubating the Δ*tsp* mutant in 0.5% sodium
404 deoxycholate (DOC); only occasionally were we able to retrieve colonies from a
405 suspension of 10^5 Δ*tsp* mutant bacteria in 0.5% DOC, which is approximately a 200-
406 fold reduction in retrieved colonies when compared to the Δ*spr*Δ*yebA* mutant for
407 the same DOC exposure. The Δ*tsp*-associated novobiocin and DOC sensitivity were
408 reverted in the Δ*spr* and Δ*spr*Δ*yebA* mutants (Table 1, Fig. 3A). We also noted that
409 the Δ*tsp* mutant had a slower growth rate in TY broth (Fig. 1G), compared to LB
410 broth (Fig. 1E), which was slightly suppressed by an Δ*spr* mutation (Fig. 1G). The
411 Δ*nlpI* mutant was also sensitized to novobiocin, a sensitization that was suppressed
412 in both the Δ*nlpI*Δ*spr* and Δ*nlpI*Δ*spr*Δ*yebA* mutants (Table 1).
413
414

415 **DISCUSSION**

416

417 **Intrinsic antibiotic-resistance barrier is associated with the outer membrane**
418 **and LPS.** In enteric Gram-negative bacteria, the outer membrane is believed to act
419 as a major barrier contributing to their intrinsic resistance to antibiotics. This
420 includes resistance to novobiocin and vancomycin that typically are only active on
421 Gram positive bacteria (34,35). Alterations that disrupt this barrier function have
422 been shown to sensitize enteric bacteria to such compounds (24).
423 Lipopolysaccharide attached to the outer membrane contributes to outer
424 membrane integrity and mutations in genes involved in LPS biosynthesis, including
425 *envA*, *rfaC* (*waaC*), and *rfaP* (*waaP*), sensitize Gram-negative bacteria
426 simultaneously to numerous antimicrobials (34,35). Outer membrane integrity can
427 also be disturbed by the expression of aberrant outer membrane proteins (36), or
428 by exposing bacteria to polymyxin B nonapetide (37,38), a compound which lacks
429 intrinsic bacteriocidal activity.

430

431 **A periplasmic enzyme, Spr, contributes to intrinsic vancomycin resistance.** In
432 this study we examined the growth and antibiotic susceptibility phenotypes
433 associated with mutations in three different periplasmic proteins involved in cell
434 wall synthesis in *S*. Typhimurium: the D,D-muramyl endopeptidases, Spr, YebA and
435 YdhO. We generated single and all combinations of double mutants lacking Spr,
436 YebA and YhdO. In this we noted that Δ*spr* and Δ*spr*Δ*yebA* mutants became
437 sensitized to vancomycin, having their MIC values decreased 8-fold respective 32-

438 fold relative to the MIC value of the wild-type (Table 1). For each of the two deletion
439 mutants, vancomycin tolerance was fully restored by ectopic expression of a wild-
440 type *spr* gene, but not by expression of an allele coding for a catalytically inactive
441 Spr (Fig. 2B). This results support the conclusion that Spr endopetidase activity
442 contributes to intrinsic vancomycin tolerance in *S*. Typhimurium. A contribution to
443 intrinsic vancomycin resistance was not a general feature of D,D-muramyl
444 endopeptidases because deleting yebA or yhdO, even in combination, did not result
445 in sensitization to vancomycin (Table 1, Fig. 3A). Nor did deletion of genes coding
446 D,D-muramyl endopeptidases MepC, NlpC or PBP7 result in vancomycin
447 sensitization (Table 1).
448
449 **Possible explanation for sensitization**. A rational explanation for vancomycin
450 sensitization associated with Δ*spr* mutations could be that they indirectly affect
451 outer membrane integrity. However, while the Δ*spr*Δ*yebA* mutant showed evidence
452 of increased outer membrane permeability (increased sensitivity to novobiocin and
453 rifampicin), the Δ*spr* mutant did not show any similar sensitization to the other
454 antibiotics tested (Table 1). Also, the antibiotic sensitization profile of the
455 Δ*spr*Δ*yebA* mutant differed from that of isogenic *ΔrfaC, ΔrfaG* and Δ*rfaP* LPS
456 mutants known to have defective outer membrane integrity, suggesting an
457 alternative mechanism for the vancomycin sensitization in the Δ*spr* and Δ*spr*Δ*yebA*
458 mutants.
459

460 **Evidence of autolysis as a mechanism**. As in *E. coli* (22), the single and double
461 mutants carrying Δ*spr,* Δ*yebA* and Δ*ydhO* had similar exponential growth rates in LB
462 broth (Fig. 1A and 1C). However, we noted that in terms of plating efficacy on LB
463 agar plates, the Δ*spr* and Δ*spr*Δ*yebA* mutants gave lower CFU yields relative to the
464 wild-type or the other mutants (Fig. 1B and 1D). In addition, the Δ*spr*Δ*yebA* mutant
465 grew to a notably smaller colony size than the wild-type (Fig. 4A). SDS-PAGE
466 analysis of supernatants from the different strains suggested autolysis of the
467 Δ*spr*Δ*yebA* mutant on plate as an explanation for the smaller colony size (Fig. 4C).
468
469 To further probe whether the autolytic behaviour of Δ*spr* and Δ*spr*Δ*yebA* mutants,
470 we assayed the release of β-galactosidase from cells exposed to antibiotics
471 inhibiting peptidoglycan synthesis. Upon exposure to increasing concentrations of
472 penicillin G or vancomycin, we noted an increased release of β-galactosidase from
473 the Δ*spr* and Δ*spr*Δ*yebA* mutants with increasing antibiotic concentrations when
474 compared to the wild-type (Fig. 5). The highest concentration of penicillin G
475 resulted in a similar plateu of β-galactosidase release wild-type as well as for Δ*spr*
476 and Δ*spr*Δ*yebA* mutant strains (Fig. 5A and 5B). The greater release of β-
477 galactosidase with increasing concentration of vancomycin by the Δ*spr* and
478 Δ*spr*Δ*yebA* mutants (Fig. 5C and 5D) most likely reflect their lower MIC to this
479 antibiotic compared to the wild-type. However, the differences between the wild-
480 type and the Δ*spr* mutant cannot be explained by a dissimilar sensitivity to
481 penicillin G resulting in different lysis, as both the wild-type and the Δ*spr* mutant
482 have the same MIC for penicillin G (Table 1).

483

484 Studies in *E. coli* have shown that prevention relies on active protein synthesis. We
485 tested this in S. Typhimurium and found, as predicted, that addition of tetracycline
486 into our β-galactosidase release assay prevented release of the enzyme both in the
487 wild-type and the Δ*spr* and Δ*spr*Δ*yebA* mutant (Fig. S3). All this makes us conclude
488 that the Δ*spr* mutants have an increased propensity for autolysis when treated with
489 either penicillin G or vancomycin.

490

491 **Suppressor screen identifies involvement of carboxypeptidase Tsp**. A screen
492 for suppressor mutations restoring vancomycin resistance in the Δ*spr* mutants
493 identified *tsp* as a candidate suppressor (Fig. 6A). Tsp is a periplasmic
494 carboxypeptidase that has been shown *in vitro* in *E. coli* to cleave MepS (Spr) in the
495 presence of the outer membrane lipoprotein NlpI (23). The suppressor character of
496 the selected Δ*tsp* mutations was confirmed by deleting *tsp* in the Δ*spr* and
497 Δ*spr*Δ*yebA* mutants and showing that the antibiotic sensitization profiles were
498 restored to wild-type levels in each of the mutants (Table 1). In addition to
499 promoting Tsp cleave Spr *in vitro,* NlpI also binds to Tsp and participates in the
500 proteolytic maturation of penicillin-binding protein 3 (PBP3) (39). Nevertheless,
501 deletion of *nlpI* did not substitute the *tsp* deletion as a suppressor for Δ*spr*-
502 associated vancomycin sensitization (Table 1) indicating a functional connection
503 between Tsp and Spr in intrinsic vancomycin tolerance without involvement of
504 NlpI.

505

506 Deletion of *tsp* also restored normal colony size, and reversed the autolytic
507 behaviour of the Δ*spr*Δ*yebA* mutant (Fig. 4). Based on the selection of suppressor
508 mutations in *tsp* we further suggest that the activity of carboxypeptidase Tsp
509 connects to the altered autolytic behaviour of Δ*spr* and Δ*spr*Δ*yebA* mutants. A caveat
510 to this suggestion would be involvement of an essential gene, not being detected in
511 our genetic screen for suppressor mutations. One such possible candidate could be
512 PBP3, which is processed by Tsp in *E. coli* (39,40). In such an event one could rather
513 envision that some of the Tsp-processed forms of PBP3 rather than Tsp itself causes
514 autolysis in the absence of Spr.
515
516 Overall, our findings suggest that the barrier maintaining intrinsic resistance to
517 vancomycinin in *S*. Typhimurium depends on the relative activities of periplasmic
518 endopeptidases. These results add to our knowledge of the dynamics of the
519 bacterial cell wall synthesis and remodelling, and importantly suggest that targeting
520 such enzymes in addition to PBPs open up a path potentiating the activity of
521 classical antibiotics used against Gram-positive bacteria.
522

523 **MATERIALS AND METHODS**

524 **Bacterial strains.** All mutant strains are isogenic to *Salmonella enterica* serovar
525 Typhimurium SR-11 (41) and are listed in Table S1. The strains LB5010 (42) and
526 ATCC 14028s were used as intermediary hosts in creating mutations. Furthermore,
527 *S*. Typhimurium strains ATCC 14028s and SL1344 were also used to host a Δ*spr*
528 mutation.

529

530 **Media and growth conditions.** Growth media included tryptone and yeast extract
531 (Sigma-Aldrich, 10 g/l respective 5 g/l) with 10 g/l of NaCl (LB medium), or without
532 NaCl (TY medium). Cultures were incubated at 37°C unless otherwise stated. When
533 needed, antibiotics were added to the growth media at the following
534 concentrations: ampicillin 100 µg/ml; chloramphenicol 25 µg/ml; kanamycin 50
535 µg/ml; tetracycline 10 µg/ml. All antibiotics were purchased from Sigma-Aldrich
536 (Sweden).

537 For determining growth curves, bacteria were incubated overnight in 2ml LB at
538 220rpm. The next day 150µl of the culture was mixed with 850µl of PBS and the
539 OD_{600nm} measured. The bacteria were then adjusted to an OD_{600nm} of 0.25. From this
540 OD_{600nm} the bacteria were then diluted 1:25 in either LB or TY broth resulting in a
541 final OD_{600nm} of 0.01. 400µl of diluted bacterial suspension was then loaded into
542 wells in a Honeycomb Bioscreen plate (OY Growth Curves AB LTd, Helsinki,
543 Finland) in three technical replicates. Uninoculated media was used as a negative
544 growth control. The Bioscreen C plate reader (OY Growth Curves AB Ltd) was set to

545 an OD of 600nm and optical density measurement was taken every 15 min with 5 s
546 of agitation before every measurement.
547
548 **PCR and oligonucleotides.** PCR was performed using an Eppendorf Mastercycler
549 Personal. Oligonucleotides were designed using the genome of *S.* Typhimurium LT2
550 as reference. For the generation of the inserts for gene knockouts the PCR was
551 performed using Phusion High-Fidelitiy PCR master mix with HF buffer (New
552 England Biolabs, USA). The cycling conditions were as following: 98°C for 1 min and
553 30 cycles of 98°C for 15 s, 54.5°C for 10 s, 72°C for 40 s, and 72°C for 2 min.

554 For routine diagnostic PCR, Phusion High-Fidelitiy PCR master mix with HF buffer
555 was used. The cycling conditions were as follows: 98°C for 1 min and 30 cycles of
556 98°C for 15 s, annealing temperature for 10 s, 72°C for elongation time, and 72°C for
557 2 min. Annealing temperature varied depending on the primer pairs used, and
558 elongation time was based on the length of the expected product (30 s per kb).
559 Oligonucleotide sequences are shown in Table S2.

560
561 **Bacteriophage transduction**. Phage P22*int* was that of Schmieger (43).
562 Transducing phages were prepared on strain LB5010 (in LB broth supplemented
563 with D-galactose (Fluka BioChemika) to 0.2% (wt/vol)), or 14028s carrying the
564 mutation of interest. The next day 200µl of chloroform was added to the culture and
565 the culture was vortexed. The culture was then centrifuged for 10 min at 18500 g to
566 create phase separation. The top phase was recovered and used to transfer the

567 genetic marker. The transduction into *S.* Typhimurium SR-11 was done by
568 incubating 20µl of the P22*int* phage containing the genetic marker with 1ml of
569 exponential phase culture. These were incubated at 37°C with shaking at 220rpm
570 for 1 hour before plating onto selective LB agar plates.

571

572 **Construction and isolation of mutants.** The antibiotic marker amplified from
573 either pKD3 (Chl^R) or pKD4 (Kan^R) was in inserted into the chromosome, to replace
574 the gene of interest, by double-stranded DNA lambda-red recombineering (44,45).
575 *S.* Typhimurium strain LB5010 containing the pSIM6, or *S.* Typhimurium strain
576 14028 containing pSIM5-tet, plasmid was grown to an OD_{600nm} of approximately 0.3
577 at 32°C with shaking at 220rpm in a water bath. To induce the lambda-red genes
578 the bacteria were transferred to a 42°C water bath shaking at 220rpm for 15 min.
579 After cooling on ice for 10 min, the cells were made electrocompetent by washing
580 with ice-cold water four times.

581 Electroporation of the resistance genes from pKD3 or pKD4 was done using a Gene
582 Pulser (Bio-Rad, USA) by mixing 25µl of electrocompetent cells and 0.5µl of purified
583 PCR product, with settings 1.8 kV, 25 F and 200Ω. Cells were recovered in 1ml of LB
584 at 32°C and 220rpm for 2 hours. After recovery the culture was spun down and the
585 pellet was spread on LB agar plates containing either chloramphenicol or
586 kanamycin to select for recombinants. The genetic marker was subsequently
587 transferred to wild-type *S.* Typhimurium SR-11 by phage P22*int* transduction from
588 either the LB5010 or 14028 mutant strains.

589 Antibiotic markers were removed from the mutants using the plasmid pCP20.
590 Briefly, pCP20 was transferred by P22*int* transduction to the recipient strain
591 containing the antibiotic marker, with selection for colonies on LB agar plates
592 containing ampicillin. Transductants were subcultured three times at 28°C on
593 selective LB agar plates. The bacteria were then subcultured three more times at
594 37°C to select for loss of plasmid and loss of antibiotic marker. The loss of the
595 antibiotic marker was confirmed by diagnostic PCR.

596

597 Isolation of vancomycin-tolerant Δ*spr*Δ*yebA* mutants was conducted by spreading a
598 LB broth culture of the Δ*spr*Δ*yebA* mutant on a vancomycin gradient TY agar plate.
599 In this, a plate of 14cm in diameter was poured in a tilted position with 37.5ml of TY
600 agar containing vancomycin at 40 μg/ml. After solidification, another plain 37.5ml
601 TY agar was poured on top of the solidified TY agar containing vancomycin, now in
602 a horizontal position. After 16 hours of incubation yielded colonies were isolated at
603 the higher concentration end of the gradient.

604

605 **Plasmid constructions and site-directed mutagenesis.** For creating plasmids for
606 genetic complementation, *spr* and *tsp* were PCR amplified using *S*. Typhimurium SR-
607 11 genomic DNA as template, using oligonucleotide primers to create suitable
608 restriction sites at each end of the amplified fragment. Enzymes used for restriction
609 digestion were SacI or EcoRI, and HindIII (New England Biolabs) while T4 DNA

610 ligase was used to ligate *spr* fragments into vector pBAD30 (46). Reaction buffers
611 were provided by the enzyme supplier. Following ligation, plasmids were
612 transformed (as above) into chemically competent *E.coli* TG1 or Top10 cells
613 (Invitrogen) from where the construct was purified and electroporated (as above)
614 into *S.* Typhimurium LB5010. The plasmid was then transferred into the correct
615 strain background of *S.* Typhimurium SR-11 by P22*int* transduction.

616

617 **Whole-genome sequencing and analysis.** Genomic DNA was prepared from
618 bacterial cultures using a Masterpure DNA Purification Kit (Epicentre). Libraries for
619 sequencing were prepared using Nextera XT sample preparation and index kits
620 (Illumina). The quality of libraries was assessed using a Tapestation 2200 (Agilent)
621 with high-sensitivity D5000 Screentape. Sequencing of the libraries was done using
622 a Miseq device (Illumina) using a 600-cycle V3 reagent kit. The sequences were
623 processed and analyzed using CLC Genomics Workbench 9.0.1 (CLC bio).

624

625 **Deoxycholate (DOC) sensitivity test.** To assay detergent sensitivity, bacteria were
626 diluted 1:100 in LB from an overnight culture, and grown in LB for 2 hours at 37°C
627 with shaking at 220rpm. The OD_{600nm} of the culture was measured, and the formula
628 $((0.484/OD_{600nm}) \times 2.1)$ used to adjust the amount of bacteria to obtain
629 approximately 10^7 CFU/ml. The bacterial suspension was then diluted 1:100 in
630 distilled water with sodium deoxycholate (DOC; Sigma-Aldrich, Sweden) freshly
631 added to a final concentration of 0.5% (wt/vol). After an incubation of 30 min at

632 room temperature, viable counts were determined from the DOC-suspension by
633 plating dilutions on LB agar plates.
634
635 **SDS-PAGE gel electrophoresis.** Polyacrylamide-bis-acrylamide gel electrophoresis
636 was conducted according to Laemmli (47), using custom made a 12.5% separation
637 (pH 8.8) and a 6% stacking gel (pH 6.8). (Thermo Scientific, Sweden). For
638 solubilisation, samples were suspended in reducing protein sample buffer (0.125 M
639 Tris-HCl pH 6.5, 3.6% SDS, 10% mercaptoethanol, 2% glycerol, and bromphenol
640 blue enough to give a deep blue color) and heated 10 minutes at 97°C before
641 application on the gel. After completed electrophoresis, gels were stained using
642 Imperial stain.
643
644 **Bacterial membrane fractionation.** The outer membrane fraction was isolated
645 according to Sabet and Schneitman (48). Bacteria from overnight LB cultures were
646 diluted 1:100 into TY broth and grown at 37°C for approximately 2 hours to mid
647 exponential phase. The OD_{600nm} of the bacterial cultures were measured and then
648 normalised. Bacteria were pelleted by centrifugation at 6000 g for 10 min, re-
649 suspended in PBS, cooled on ice, and disrupted by equal numbers of 10 s sonication
650 pulses until the suspensions visibly cleared. Bacteria were removed by low–speed
651 centrifugation (1500 g), after which the membrane fraction was pelleted by high-
652 speed centrifugation (18500 g, 10 min). The waxy brownish pellet was then re-
653 suspended in 50mM Tris-HCl buffer containing 10mM $MgCl_2$ and 0.5% Triton X-
654 100. The membrane fraction was again pelleted by centrifugation (18500 g, 10

655 min), and the re-suspension and high-speed centrifugation steps were repeated.
656 The final colourless pellet was then
657
658 **Measuring bacterial lysis.** To quantify bacterial lysis, colonies grown overnight on
659 LB agar plates (18 hours at 37°C) were suspended in PBS, with adjustment to the
660 same OD_{600nm}. Bacteria were removed from the suspension by centrifugation (2 x
661 18500 g). The supernatant was made 10% (wgt/vol) in trichloroacetic acid (Sigma-
662 Aldrich), and incubated at -20°C overnight. The samples were then thawed, mixed,
663 centrifuged at 18500 g and washed with ice-cold acetone. The acetone phase was
664 carefully removed by pipetting and the tubes dried at 37°C for 15 min. The samples
665 were then processed for SDS-PAGE analysis as described above.
666
667 **Disk diffusion.** Bacteria were grown overnight in LB broth at 37°C and 220rpm.
668 The overnight culture was diluted 1:100 in LB broth and grown for 2 hours at 37°C
669 and 220rpm. The OD_{600nm} was measured of the 2 hour culture and the formula
670 $((0.484/OD_{600nm}) \times 2.1)$ was used to calculate the amount of bacteria needed for 10^6
671 CFU/ml. 3 x 10^5 CFU/ml bacteria were then spread on large (⦸13.7 cm) TY agar
672 plates and the antibiotic disks were placed on top of the bacteria and the plates
673 were incubated overnight at 37°C. The next day the diameters of the inhibitory
674 zones were measured.
675
676 The disk, 6mm in diameter, were custom-made out of Whatman 3 paper. Each disk
677 was infused with 5µl of an antibiotic. Concentrations of the antibiotics used were;

678 tetracycline 10 µg/ml, vancomycin 20 µg/ml, rifampicin 10 µg/ml, polymyxin B 10
679 µg/ml, novobiocin 10 µg/ml, and penicillin G 10 µg/ml (Sigma-Aldrich, Sweden).
680 Every antibiotic was dissolved in water except rifampicin, which was dissolved in
681 DMSO.
682
683 **MIC determination.** Bacteria were grown overnight in LB broth at 37°C and
684 220rpm. The overnight culture was diluted 1:100 in LB broth and grown for 2 hours
685 at 37°C and 220rpm. The OD_{600nm} was measured of the 2 hour culture and the
686 formula (($0.484/OD_{600nm}$) x 2.1) was used to calculate the amount of bacteria
687 needed for 2x 10^4 CFU/ml in TY broth. This dilution was subsequently pipetted into
688 a 96-well plate, containing TY broth with either vancomycin or penicillin G,
689 resulting in a final concentration of 10^4 CFU/ml bacteria. The highest concentration
690 for the vancomycin MIC serie was 100 µg/ml and 50 µg/ml for penicillin G. Each
691 serie was diluted 1:2 from the highest concentration.
692
693 **Drop-on-lawn.** Bacteria were grown overnight in LB broth at 37°C and 220rpm.
694 The overnight culture was diluted 1:100 in LB broth and grown for 2 hours at 37°C
695 and 220rpm. The OD_{600nm} was measured of the 2 hour culture and the formula
696 (($0.484/OD_{600nm}$) x 2.1) was used to calculate the amount of bacteria needed for 10^6
697 CFU/ml. From 10^6 CFU/ml a 1:2 serial dilution series was made into 1ml PBS. From
698 each dilution a 5µl droplet was pipetted onto TY agar plates containing none, 20
699 µg/ml, or 40 µg/ml vancomycin and the TY agar plates were incubated overnight at

700 37°C. For genetic complementation tests TY agar plates were supplemented with
701 0.02% (wgt/vol) L-arabinose (Sigma-Aldrich, Sweden).
702
703 **Viable counts from broth.** Viable counts were enumerated by taking bacteria from
704 a replicate honeycomb in the bioscreen during the growth curves. 100µl was
705 removed from one well in the honeycomb and added to 900µl PBS at time points 2.5
706 hours, 6 hours, and 11 hours of the OD_{600nm} was measured. The measurements were
707 used to calculate how much bacteria to take for 10^6 CFU/ml. This suspension was
708 further 1:10 serially diluted to an estimated 10^3 CFU/ml from which 100µl was
709 spread in triplicates for 10^2 CFU on each LB agar plate. The LB agar plates were
710 incubated overnight at 37°C and the CFU for the triplicates were calculated and the
711 average was obtained and normalised to wild-type.
712
713 **Viable counts from plate.** Viable counts were enumerated by growing bacteria
714 overnight at 37°C on a LB agar plate. Individual colonies from the LB agar plates
715 were suspended in PBS and the OD_{600nm} was measured. The measurement and
716 following protocol was treated as above.
717
718 **β-galactosidase release assay**. A phage lysate was prepared from LB5010 carrying
719 pKTH3088 (49), which is a pACYC184 derivative containing the *E.coli lacZ* gene
720 from pMC1871 (50). This P22 lysate was used transfer the pKTH3088 to *S.*
721 Typhimurium SR-11 strains. To assay for the release of LacZ, bacteria were grown
722 overnight in LB broth at 37°C and 220rpm. The overnight culture was diluted 1:100

723 in TY broth and grown for 2 hours at 37°C and 220rpm to mid exponential phase,
724 whereafter bacteria were removed by centrifugation (18500 g), and LacZ activity
725 measured from the supernatants and whole cells according to Miller (51).
726
727 **Statistical analysis.** GraphPad Prism v6.0g (GraphPad Software, Inc., USA) was
728 used for statistical analysis. The statistical test for Fig. 1, Fig. 3A, Fig. 5B and Fig. S2
729 was one-way ANOVA with Dunnett's correction for multiple samples. The statistical
730 test for Fig. S4 was a t-test comparing each concentration to each other.
731

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741
742

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