Metabolic homeostasis controls the diversity of cooperative swarming in pathogenic bacteria

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Abstract

Many bacteria have an incredible ability to swarm cooperatively over surfaces. But swarming phenotypes can be quite different even between strains of the same species. What drives this diversity? We compared the metabolomes of 29 clinical *Pseudomonas aeruginosa* isolates with a range of swarming phenotypes. We identified that isolates incapable of secreting rhamnolipids—a surfactant needed for swarming—had perturbed tricarboxylic acid (TCA) cycle and amino acid pathways and grew exponentially slower in glycerol minimal medium. Analysis of the metabolome signatures and simulations using a genome-scale model led to a mechanism which joins these observations: Strains subject to higher oxidative stress levels grow slower and shut down rhamnolipids secretion, a carbon overflow mechanism possibly to direct carbon resources towards costly stress response pathways to maintain cell viability. *In vitro* experiments confirmed that rhamnolipid non-producers deal worse with oxidative stress, linking intracellular redox homeostasis—a individual-level trait—to swarming—a population-level behavior. This mechanism helps explain the metabolic constraints on bacteria when secreting byproducts to interact with others—competitively and cooperatively—in microbial communities.

Significance

Swarming motility has been associated with virulence of many human bacterial pathogens. The pathogen *Pseudomonas aeruginosa* swarms by cooperatively secreting surfactants called rhamnolipids to lubricate surfaces. To understand why some *P. aeruginosa* strains swarm and others do not, we combined metabolomics, computational modeling and *in vitro* experiments to study the different swarming behaviors of 29 isolates of *Pseudomonas aeruginosa* obtained from infected patients. We found that strains can only produce rhamnolipids if they can maintain redox homeostasis. We propose that single cells must have low internal redox stress

levels before they can produce rhamnolipids, which work as an overflow of carbon metabolism into a cooperative secretion that brings a fitness benefit to the entire swarming population. This mechanism links single-cell physiology and a population-level cooperative behavior key to the fitness and virulence of *P. aeruginosa*, a major source of hospital acquired infections.

Synopsis

This study combined metabolomics, computational modeling and experiments to explain the swarming diversity in *Pseudomonas aeruginosa*, yielding new insights on the genetic and metabolic controls of bacterial swarming behavior

- Rhamnolipid secretion is necessary, but insufficient, for swarming
- *P. aeruginosa* strains unable to produce rhamnolipids in the glycerol minimal medium have perturbed metabolism, slow growth and elevated oxidative stress
- Rhamnolipid producers cannot produce rhamnolipids in succinate as the sole carbon source which causes greater ROS (reactive oxygen species) production
- The requirement of redox homeostasis for rhamnolipid production ensures that the overflow of precious carbon for population-level benefit is prudently controlled
- This study helps linking single cell physiology with collective behavior key to the fitness and virulence of a pathogenic bacterium.

Introduction

Swarming enables millions of bacteria to travel together across centimeter-long distances in a few hours (Deforet *et al*, 2014). This cooperative trait found typically in the phyla Alpha-proteobacteria and Gamma-proteobacteria is a complex phenotype that requires the coordinated expression of many genes: Swarming requires flagella or pili to propel the cells, cell-cell interactions to maintain population integrity during migration, and the collective secretion of biosurfactants to lubricate the surface (Kearns, 2010). Studies over the past two decades revealed many molecular details of swarming motility through genetic (e.g., knockouts, transgenesis) and environmental (e.g., nutrient composition and viscosity of culture medium) perturbations (Köhler *et al*, 2000; Mattingly *et al*, 2018) as well as through experimental evolution (van Ditmarsch *et al*, 2013). Some genes that modulate swarming impact key cellular activities including tricarboxylic acid (TCA) cycle and stress response (Inoue *et al*, 2007; Tremblay & Déziel, 2010) and therefore have pleiotropic effects. Despite these insights, we lack a systems-level understanding of this cooperative bacterial behavior (Kim & Surette, 2004; Inoue *et al*, 2007).

Pseudomonas aeruginosa—an opportunistic human pathogen and a major cause of hospital-acquired infections (Klebens *et al*, 2007)—has a remarkable swarming ability that produces long straight segments (tendrils) in its fractal-like swarming pattern. Similar to other swarming species, *P. aeruginosa* requires both flagella and pili to move, the LasR-LasI and RhlR-RhlI quorum sensing systems to communicate within population, and the ability to produce rhamnolipids to lubricate the surface (Köhler *et al*, 2000). Rhamnolipids are a mixture of biosurfactants that consist of 3-(3-hydroxyalkanoyloxy) alkanolic acids (HAAs), mono-rhamnolipids and di-rhamnolipids (Abdel-Mawgoud *et al*, 2010). RhlA is the only enzyme required to drive the conversion of fatty acid biosynthesis intermediates (β -

hydroxyacyl-ACP) to HAAs (Zhu & Rock, 2008), whereas RhlB and RhlC each add one molecule of rhamnose to the HAAs to produce mono-rhamnolipids and di-rhamnolipids in sequential steps (Chong & Li, 2017). The genes encoding RhlA and RhlB are located in the same operon *rhlAB*, which is regulated by the quorum-sensing cascade headed by LasR-LasI and followed by RhlR-RhlI (Medina *et al*, 2003). Other than quorum-sensing signals, *rhlAB* expression is also controlled by nutrient cues such as an excess of carbon source (Xavier *et al*, 2011; Boyle *et al*, 2015; Mellbye & Schuster, 2014). The combination of quorum-sensing and metabolic signals is called metabolic prudence; it prevents cheating by rhamnolipid non-producers such as the $\Delta rhlA$ mutant because it prudently ensures that rhamnolipids are only produced at the right time when the production benefits outweigh its costs. Strains that produce rhamnolipids too soon or overproduce rhamnolipids, such as a strain engineered with the inducible $P_{BAD}\text{-}rhlA$ construct, have less carbon available for growth and are susceptible to cheating by non-producers (Xavier *et al*, 2011; de Vargas Roditi *et al*, 2013).

Besides the role of metabolic prudence, which regulates rhamnolipid production and makes swarming robust to cheating, other aspects of metabolism might have influenced the evolution of swarming across the *P. aeruginosa* phylogenetic tree. Metabolism is the currency of all physiological processes that support life (Smith & Morowitz, 2004) and a major determinant of social behavior (Biro & Stamps, 2010). A previous study (Boyle *et al*, 2017) compared the intracellular metabolites of the laboratory strain PA14 and several mutants of that strain with different swarming phenotypes. The $\Delta rhlA$ mutant can neither swarm nor produce rhamnolipids, but it had less than 10 metabolites perturbed compared to the wild-type. The $\Delta cbrA$ mutant, which lacks a global regulator of carbon-nitrogen balance, had impaired swarming and lower growth rate with dozens of perturbed metabolites. Notably, the $\Delta cbrA$ mutant still had the flagella needed to move and secreted even more rhamnolipids than the wild-type. More interestingly, its swarming phenotype could be rescued by

compensatory point mutations in the RNA chaperone *hfq* through rewiring of metabolic network to reconstruct a unique metabolome which was distinct from both the $\Delta cbrA$ and the wild-type (Boyle *et al*, 2017).

Here we leveraged a collection of 29 clinical isolates of *P. aeruginosa* that we previously isolated from hospitalized cancer patients in Memorial Sloan-Kettering Cancer Center (MSKCC) (Yan *et al*, 2017). These strains span a wide range of swarming phenotypes; however, they evolved independently in environments that are largely unknown to us and exhibit great genetic diversity, which makes it hard to pin down the exact genes responsible for their different swarming behaviors. Since the differences cannot be solely explained by genetics, here, in this study, we combined genomics with metabolomics, growth curve analysis and computational models to study the metabolic differences underlying the observed swarming diversity. Swarming requires rhamnolipid production; here we found that rhamnolipid-producing strains have reduced TCA cycle activity, increased levels of amino acids, and slower exponential-phase growth rate compared to the non-producing strains. We further provided both computational and experimental evidences showing that these metabolic changes are likely caused by a higher oxidative stress. This finding suggests that rhamnolipid production is a form of overflow metabolism that is only activated in strains with sufficient capability to detoxify reactive oxygen species (ROS) produced by energy metabolism and maintain redox balance. This mechanism links redox homeostasis with swarming cooperation, highlighting the importance of metabolism in the evolution of a social behavior.

Results

Phylogenetic analysis suggests loss of rhamnolipid production drove loss of swarming in some isolates. We started by analyzing the shapes of swarming colonies of clinical isolates

and three common reference strains whose genomes vary widely (Fig. 1). We used image analysis to measure six morphological features of the colonies, including perimeter, maximum length, area, circularity, skeleton longitude and eccentricity (see **Materials and methods** for their definitions). A principal component analysis of these morphological features revealed that the maximum length and circularity captured the most variability of the shape diversity in our tested strains (Supplementary Fig. 1). We quantified the swarming ability of all strains using a linear combination of the two features but found no significant correlation of the swarming scores with the phylogeny inferred from their core genomes (Moran's I test, p-value=0.93). Since production of rhamnolipids is required for swarming motility, we measured the ability of strains to produce rhamnolipids in a synthetic media with glycerol as the sole carbon source (Boyle *et al*, 2015). As expected, all strains able to swarm produced rhamnolipids. Not all rhamnolipid producers swarmed, though, which confirms that rhamnolipids are necessary but not sufficient for swarming (Caiazza *et al*, 2005; Déziel *et al*, 2003). Similar to swarming phenotype, the ability to produce rhamnolipids, semi-quantitatively classified as non-, mild- and strong-producers (**Materials and Methods**), was also uncorrelated with the phylogeny (Fig. 1B, Moran's I test, p-value=0.14). To better assess the link between swarming and rhamnolipid production, we built an evolutionary model to reconstruct their ancestral states along the phylogenetic tree. The model predicted that the common ancestor of all the strains can both swarm and produce rhamnolipids. This prediction suggests that the diversity of swarming found across the isolates was caused by some strains losing their ability to swarm or produce rhamnolipids, not by concurrent lineages evolving those functions independently. The model also suggests that rhamnolipid production is more conserved than swarming: the immediate ancestors of all non-producers are inferred to be rhamnolipid producers (Supplementary Figure 2A). By contrast, the loss of swarming occurred earlier than that of rhamnolipid production in several lineages (Supplementary

Figure 2B), a result which is also expected because swarming depends on other factors besides rhamnolipid production (Kearns, 2010).

The loss of rhamnolipid production is partially explained by the absence of genes. Since the phylogeny inferred from core genes was unable to explain the observed diversity of rhamnolipid production, we asked whether accessory genes, particularly those only missing in rhamnolipid non-producers, could explain the diversity. Here we focused on the comparison between rhamnolipid producers (both mild and strong rhamnolipid-producing strains; the same below) and non-producers, rather than between swarmers and non-swarmers, because (1) the latter phenotype is harder to determine non-arbitrarily and (2) rhamnolipid production is itself a predictor of swarming (all rhamnolipid non-producers lose swarming). We then compared the genomic sequences between the 8 rhamnolipid non-producers and the remaining producers using reciprocal blast to explore the genetic features that may be associated with rhamnolipid production. We identified 304 genes (Supplementary File 1) only absent in those non-producers that may directly contribute to the loss of rhamnolipid production (89 well annotated genes are listed in Table 1): F5677 lacks the flagella motor switch protein *fliM*, and *qscR*—a quorum-sensing control repressor; W36662 lacks the type IV pili assembly protein *pilC* and its sensor kinase *pilS*; and F63912, W36662, W60856 lack three quorum-sensing genes *lasR*, *rhlR*, and *rhlI*. It was previously shown that the single-gene knockout mutants $\Delta fliM$, $\Delta pilS$, $\Delta rhlR$ and $\Delta rhlI$ (Köhler *et al*, 2000; Overhage *et al*, 2007; Kamatkar & Shrout, 2011), but not $\Delta pilC$ (Luo *et al*, 2015), displayed strong swarming-deficient phenotypes. The mutant of a two-component response regulator gene *pirR* that is missing in H27930 was found to repress swarming in multiple culture media (Kollaran *et al*, 2019). S86968 lacks *nfxB*, whose mutation caused global dysregulation of physiology and metabolism in *P. aeruginosa* including impaired swarming (Stickland *et al*, 2010). Notably, genes encoding rhamnolipid biosynthesis (*rhlA*, *rhlB*, *rhlC*)

are intact and contain no mutation in all non-producers, indicating that the mutations that lead to the loss of rhamnolipid production are linked to the regulation of the pathway, either in other regulatory genes (in coding or non coding regions) or in non-coding regions of the biosynthetic genes.

Besides these functions that are directly linked to rhamnolipid production and swarming, genes involved in carbon catabolism, biofilm, chemotaxis, antibiotic resistance, virulence and redox regulation were also found missing in rhamnolipid non-producers. M55212 misses the catabolite repression control gene *crc*, although a *crc* transposon mutant is able to swarm as well as the wild-type (Yeung *et al*, 2011). H27930 lacks the methyl-accepting chemotaxis genes *pctA* and *pctB* and the biofilm biosynthesis gene *pelA*. Both M1608 and S86968 lack the pyridoxal phosphate (vitamin B₆) biosynthetic gene *pdxA* and the *hcnABC* genes coding for the virulence factors hydrogen cyanide (Pessi & Haas, 2000). F5677 cannot express PhzA2 for biosynthesis of phenazine—a well-known virulence factor. The absence of *amrB* that encodes a membrane protein of the AmrAB-mediated efflux system in M1608 suggests that the strain may be sensitive to the aminoglycoside antibiotic (Westbrock-Wadman *et al*, 1999). The missing genes for redox regulation include *soxR* (absent in F5677)—a redox sensitive transcriptional regulator, *katE* (absent in M1608)—a catalase that degrades H₂O₂ (Mulvey *et al*, 1988), and *gor* (absent in M1608)—a flavoprotein that oxidizes glutathione, which plays an important role in protection against H₂O₂ damage (Perry *et al*, 1991).

Rhamnolipid producers grow faster in glycerol. The missing genes in the 8 non-rhamnolipid-producers (Table 1) include several global transcriptional regulators (e.g., *rhIR*, *lasR*, *crc*, *nfxB*, *soxR*) that are broadly important for cell metabolism and growth. Their absence may cause growth-independent or -dependent metabolic effects on rhamnolipid production and swarming. We first tested whether growth is a predictor for the observed

diversity. We monitored growth curves of all our strains over 48h in the same glycerol media that we had used to measure rhamnolipid production. These growth curves exhibited markedly different lag times and exponential growth-rates (Fig. 1C), but a high similarity was observed among 6 out of 9 swarming strains (Supplementary Fig. 3). However, some rhamnolipid producers clustered with non-producers, suggesting that rhamnolipid production cannot be classified by the entire growth curves and more sophisticated methods to extract local growth curve features were needed.

We then used both unsupervised and supervised learning approaches to identify features in the growth curves associated with rhamnolipid production. For the unsupervised approach, we used non-negative matrix factorization (Lee & Seung, 1999) which decomposed each growth curve as a weighted sum of three basis functions (i.e., features). Although the growth curves of the rhamnolipid producers (orange lines) and non-producers (blue lines) largely overlapped (Fig. 2A), there was a significant difference in the weights associated with basis 1 between the two groups (Fig. 2B), which suggests a link between rhamnolipid production and bacterial growth. To pinpoint features associated with rhamnolipid production, we divided each growth curve into three phases based on its shape (Fig. 2C and Supplementary Fig. 4) and defined 7 quantitative features to characterize each growth phase (Fig. 2D and Supplementary File 2). Using Random Forest classification, we found that the top two features with the highest explanatory power were the maximum and averaged specific growth rates in phase I when growth speeds up (Fig. 2E). Considering the majority of rhamnolipids are produced in phase II when growth slows down (Boyle *et al*, 2015), our finding revealed a strong temporal link between exponential growth rate in phase I and rhamnolipid production in phase II. We hypothesized that the link might be mediated by metabolic homeostasis: its disruption could slow down exponential growth in phase I and disfavor rhamnolipid secretion

in phase II by saving precious carbon resources for cell maintenance and stress responses under metabolic imbalanced conditions.

Rhamnolipid non-producers have perturbed TCA cycle and amino acid pathways.

Similar to our growth curve analysis, we adopted both unsupervised and supervised learning approaches to test the associations of intracellular metabolite levels with swarming and rhamnolipid production. We profiled the intracellular metabolome of all our strains in the same glycerol minimal medium (except for two rhamnolipid non-producers M55212 and F23197 which grew too slow) during the transition between phase I and phase II when rhamnolipid production begins (**Materials and Methods**). Using LC-MS (Liquid chromatography-Mass spectrometry) we identified a total of 92 compounds spanning a wide range of abundances (Supplementary Fig. 5A), including 67 metabolites whose identities are known and abundances vary significantly across all the tested strains (Fig. 3A). After data normalization and imputation (Supplementary Fig. 5B), hierarchical clustering showed the consistency of data across all three replicates for each strain (Fig. 3B), except for one replicate of H47921 (sample 25, an outlier removed from further analysis). The similarity among the metabolomics data yielded three major clusters with one cluster (middle cluster in Fig. 3B) containing all strong swimmers (strain name in red) and only rhamnolipid producers. However, no clear metabolic pattern was found among non-producers: Two non-producers were grouped in the left cluster and 4 in the right cluster, and they all had metabolomics profiles similar to producers within the same cluster.

The lack of a clear metabolomic profile for rhamnolipid non-producers led us to search for specific differential metabolites using supervised methods. We fitted our metabolomic data (explanatory variables) to categorized rhamnolipid production (response variable) using Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA) (Cloarec *et*

al, 2005). The OPLS-DA produced a reasonable fit ($R^2 = 0.82$, $Q^2 = 0.66$, p -value = $5e-4$) with the single predictor that separated rhamnolipid non-producers from producers explaining only 5% of the variance across all data (Fig. 4A). The remaining 95% of the variance was explained by three orthogonal components that capture variation within both groups, again showing that there is a high overlap between the metabolomic profiles of rhamnolipid producers and non-producers. The loading values of the predictive component reflect how much each compound contributes to the separation between rhamnolipid producers and non-producers with negative loadings indicating higher metabolite abundance in non-producers (Fig. 4B and Supplementary Fig. 6).

We then used a univariate Mann-Whitney U test to identify the metabolites differentially abundant in producers and non-producers. We found 24 such metabolites (bars with black outline in Supplementary Fig. 6) and these metabolites were then used to identify pathways perturbed in non-producers using the FELLA algorithm (Picart-Armada *et al*, 2017, 2018) (Supplementary Table 1). The most discriminative pathways were the TCA cycle and amino acid metabolism (Fig. 4B). 3 out of 6 metabolites in the TCA cycle—fumarate, succinate and citrate—were significantly changed: Fumarate and malate showed a positive loading value, while citrate, succinate, and to a less extent cis-aconitate and alpha-ketoglutarate were negative (Fig. 4B). Pyruvate remained relatively constant across all the strains (Fig. 3A), implying that the differential responses in the TCA cycle were independent from the changes in its upstream central carbon metabolism.

Besides the TCA cycle metabolites, the majority of annotated compounds in the metabolism of branched chain amino acids (leucine/isoleucine, valine) and sulfur-containing amino acids (cysteine/methionine) had higher abundance in rhamnolipid non-producers relative to producers. In fact, with the exception of glutamate, proline and tryptophan, all other amino

acids detected in our metabolomics data (threonine, alanine, aspartate, glutamine, cysteine, lysine, histidine, tyrosine, and arginine) were higher in non-producers (Supplementary Fig. 6). A striking exception to this trend was formylmethionine (fMet), which had lower abundance in non-producers. Diminished level of fMet had also been found in the $\Delta rhIA$ mutant of *P. aeruginosa* (Supplementary Fig. 7) (Boyle *et al*, 2017). Since $\Delta rhIA$ mutant grows with a similar growth rate as the wild-type (van Ditmarsch & Xavier, 2011) the link between fMet and rhamnolipid production should not be simply due to a growth defect.

Metabolome signature and computer simulations link high oxidative stress to lower

rhamnolipid production. So far our metabolomics data show that the non-producers have perturbed TCA cycle activity and grow slowly. As shown in Fig. 4C, the TCA cycle harbors five enzymes with Fe-S clusters (aconitase A, aconitase B, succinate dehydrogenase, fumarase A, fumarase B (Py & Barras, 2010)) and thus represents one of the most vulnerable pathways subject to attacks of ROS. It is likely that higher oxidative stress in the non-producers reduces flux through the TCA cycle, which would explain their slower growth. The significantly opposite association of succinate and fumarate we found in rhamnolipid production indicates the reduced activity of succinate dehydrogenase (SDH) under oxidative stress conditions. SDH is a membrane-bound dehydrogenase linked to the respiratory chain—a major site of ROS production in the cell—and also a member of the TCA cycle that catalyzes the oxidation of succinate into fumarate (Hederstedt & Rutberg, 1981). Since SDH contains [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters (Ayala-Castro *et al*, 2008), ROS that damages Fe-S clusters could decrease SDH activity *in vivo*. The metabolomics data also showed that some intermediate metabolites in amino acid biosynthetic pathways, which are also substrates of Fe-S containing enzymes, accumulated in the non-producers (Fig. 4C). For example, the glutamate synthetase that consists of a large chain (encoded by *gltB*) and a small chain (encoded by *gltD*). Both subunits contain Fe-S clusters and catalyze the production of

glutamate from oxoglutarate and glutamine. We found slightly higher oxoglutarate and glutamine as well as lower glutamate in the non-producers. These observations collectively suggest that the rhamnolipid non-producers might have higher oxidative stress levels than the producers.

These reasons suggest that the cause for impaired rhamnolipid production might be that non-producing strains suffer from high oxidative stress. We then sought to use a whole-genome reconstruction model to study how oxidative stress could lower the flux of carbon available to rhamnolipid production. We modified a high-quality genome-scale model of *Pseudomonas* metabolism, iJN1411 (Nogales *et al*, 2017, 2020), to simulate growth in the culture medium used in our metabolomics experiments with glycerol and ammonium as the sole carbon and nitrogen sources. Rhamnolipids are produced when carbon is in excess (Boyle *et al*, 2015). In this model rhamnolipids secretion occurs when the carbon-to-nitrogen (C:N) ratio exceeds a 6.3 threshold (Supplementary Fig. 8). We set C:N to 10.0, which exceeds the minimum threshold, and we varied the redox stress level by changing the flux levels of three redox molecules: NADH (reduced nicotinamide adenine dinucleotide), NADPH (reduced nicotinamide adenine dinucleotide phosphate) and GSH (reduced glutathione); these molecules are responsible for the bulk of cellular electron transfer and likely to be the main sources of ROS (Xiao & Loscalzo, 2019). Although redox stress is ultimately caused by high ROS levels, not fluxes, and ROS was not an explicit variable in our model, we assumed that ROS can perturb intracellular metabolic fluxes, particularly the fluxes of redox molecules, and asked how cell growth and rhamnolipid secretion respond to these ROS-mediated indirect perturbations.

For all three redox molecules, we found that the maximum growth rate was maintained at an intermediate flux range (redox homeostasis, white area) while any flux rate outside the range

(gray shading) gradually reduced growth rate (Fig. 5, upper panels). What generally accompanied the compromised growth rate was the abrupt shutdown of secretion of rhamnolipid precursors (HAA), mono- and di-rhamnolipids as well as many (but not all) central carbon metabolites, except for extremely small GSH flux (Fig. 5, lower panels). Importantly, reducing any of these secretion fluxes did not reduce the growth rate, nor did it provide growth benefits (i.e., each individual flux could be as low as zero without reducing maximum growth rate). This observation agrees with the data that the *ArhLA* mutant of *P. aeruginosa* type strain PA14 has the same grow rate as its wild-type strain in the same glycerol minimal medium (van Ditmarsch & Xavier, 2011). Notably, most of the overflowed carbon-rich molecules, including HAA, mono- and di-rhamnolipids, were only secreted when the growth rate maintained its maximum value under excess carbon. The computational inference that rhamnolipids, as well as other central carbon metabolism intermediates, can be secreted under maximum growth suggests that *P. aeruginosa* cells have the flexibility to overflow carbon through alternative metabolic pathways without compromising growth. The fact that *P. aeruginosa* maintains the secretion of rhamnolipids among these options supports that rhamnolipid secretion was favored by natural selection for reasons other than improving single-cell fitness. Collectively, these simulations support a link between rhamnolipids secretion and redox stress, where fast growth and rhamnolipid secretion are both metabolically constrained by cellular redox homeostasis.

Experiments support the link between growth, oxidative stress and rhamnolipid production. We validated the link between oxidative stress and rhamnolipid production by two additional experiments. In the first experiment, we monitored growth and rhamnolipid production of all our clinical isolates when they grew in the same minimal medium but with succinate as the sole carbon source (Supplementary Fig. 9). Since succinate enters the carbon metabolism directly through the TCA cycle, we expected that, compared to growth in

glycerol, the initial TCA cycle flux would be much faster and generates higher ROS level that impairs the sustained cell growth and rhamnolipid secretion. Indeed, none of the strains that produced rhamnolipids in glycerol produced rhamnolipids in succinate. We similarly extracted 7 phase-dependent growth curve features of our strains grown in succinate (Supplementary File 3) and compared their values to the same features measured in glycerol (Supplementary Fig. 10). The comparison showed that all strains had shorter lag phases and grew exponentially faster in succinate. The strains reached much lower cell densities even though the succinate medium had the same initial molar concentration of carbon as the glycerol medium. More interestingly, phase II was almost absent in succinate compared to glycerol. The strains all switched abruptly from phase I to phase III, suggesting that cells might have accumulated more oxidative stress during phase I, which caused a sudden stop in growth as well as carbon overflow.

In the second experiment, we monitored the hydrogen peroxide (H_2O_2) level when growing our *Pseudomonas* strains in the glycerol minimal medium (Fig. 6A,B). H_2O_2 is a representative ROS that can diffuse freely between cell and the environment. By measuring the fluorescent signal intensity produced by the Amplex assay (**Materials and Methods**), we found a baseline production of H_2O_2 in the absence of cells, possibly due to photooxidation of Amplex Red reagent (Zhao *et al*, 2012). When cells were added, *Pseudomonas* strains can degrade H_2O_2 by producing many antioxidant enzymes including catalases, glutathione reductase, NADH peroxidase and cysteine-based peroxidases (Mishra & Imlay, 2012). We computed the signal intensity difference between cell culture and baseline as a proxy of the ability of each strain to remove H_2O_2 and broadly combat oxidative stress. We saw that all strains except for M1608 quenched the signal over the first 18 hours (Fig. 6B). Most importantly, the non-producers (blue lines) removed less H_2O_2 than mild rhamnolipid producers (green lines) and strong producers (red lines), supporting our hypothesis that non-

producers are worse at removing ROS. Unsurprisingly, the worst strain in this assay was M1608, which lacks *katE* and *gor* (Table 1), both of which are important for H₂O₂ degradation. To factor out the possibility that the non-producers performed worse due to lower cell density, we calculated the H₂O₂ removal rate per cell and observed similar relative trend of H₂O₂ degradation between non-producers and producers (Fig. 6C). Formally, we used a linear mixed-effect model to test the effect of rhamnolipid production on per-cell H₂O₂ removal rate (**Materials and Methods**). The effect size of mild and strong producers relative to the non-produces are 1.25x and 1.41x stronger respectively with both p-values < 0.001, confirming that rhamnolipid producers deal better with oxidative stress.

Discussion

Our data comparing growth phenotypes and metabolomes posit a model to explain the diversity of swarming phenotypes we saw in *P. aeruginosa* isolates (Fig. 7): Some strains can produce rhamnolipids because they maintain redox homeostasis due to their greater abilities to degrade ROS. Rhamnolipid non-producers by contrast had disrupted redox homeostasis, causing slower growth with reduced TCA cycle activity and increased amino acid concentration. The impaired ability to degrade ROS by non-producers is possibly caused by loss of genes that encode ROS detoxification functions (e.g., M1608 lacks *katE* and *gor*). Consistently, it was reported that OxyR—oxidative stress regulator that controls expression of antioxidant genes—is required for swarming and rhamnolipid production (Vinckx *et al*, 2010).

All our clinical strains were isolated from hospitalized patients, a host-associated environment where we may expect redox stresses such as ROS imposed by the immune system in their fight against pathogens (Puertollano *et al*, 2011) and by antibiotics treatment (Zhu *et al*, 2019). Non-producers of rhamnolipids seem less capable of dealing with oxidative

stresses, and it remains unclear what selected for this loss of function *in vivo*. Rhamnolipid production was, nonetheless, relatively frequent across the *P. aeruginosa* phylogenetic tree. The genes for rhamnolipid biosynthesis (*rhlA*, *rhlB*, *rhlC*) remained intact across all of our clinical isolations, even in non-producers. This suggests that the loss of rhamnolipid production in some strains may have resulted from broader metabolic adaptations such as oxidative stress responses. In support of our model we saw that rhamnolipid producers could quench oxidation faster than non-producers (Fig. 6). Accumulation of oxidative stress due to slow removal can reduce SDH activity and perturb TCA cycle (Fig. 7). Others have shown that *P. aeruginosa* decreases expression of the key quorum-sensing genes (*LasI*, *LasR*, *RhlI*, *RhlR*, *PqsA* and *PqsR*) in response to oxidative stress caused by exposure to H₂O₂ (Mohamed *et al*, 2020). Considering quorum sensing regulates hundreds of genes (Lequette *et al*, 2006), shutting down the cooperative traits that are under quorum-sensing regulation—including rhamnolipid secretion—could save precious resources for oxidative stress responses.

We also saw that rhamnolipid non-producers had significantly lower levels of fMet than producers. Interestingly, this also happens in the $\Delta rhlA$ mutant compared to its wild-type strain PA14 (Boyle *et al*, 2017). fMet plays a role in translation initiation, and in the quality control mechanisms that degrade misfolded proteins (Piatkov *et al*, 2015). *Bacillus subtilis* lacking Formyl-Methionine Transferase (FMT)—the enzyme attaching a formyl group to methionine loaded on tRNA^{fMet}—are more sensitive to H₂O₂ and defective in swarming (Cai *et al*, 2017). The link between fMet, swarming and oxidative stress is intriguing and warrants further research.

Only the strains that grew fast on glycerol could produce rhamnolipids in that medium. This supports the notion that rhamnolipid production is a form of overflow metabolism. Overflow metabolism is associated with fast growth such as bacteria under conditions of high glucose

consumption. It occurs when cells simultaneously operate on both energy-efficient (e.g., respiration) and -inefficient (e.g., fermentation) pathways and secrete metabolic byproducts that could otherwise be used for catabolism or anabolism. This phenomenon has been observed in different systems: aerobic fermentation in *Escherichia coli* (Farmer & Jones, 1976), the Crabtree effect in *Saccharomyces cerevisiae* (De Deken, 1966) and the Warburg effect in cancer cells (Vander Heiden *et al*, 2009). Many molecular mechanisms have been proposed and validated to account for the seemingly wasteful usage of low energy-yield pathway in presence of a higher-energy-yield alternative (Basan *et al*, 2015; Senk *et al*, 2017). For example, the respiratory flux that would be required for fast growth without fermentation could accumulate NADH that could not be recycled to NAD⁺ by respiration alone, because of a limited surface/volume ratio (Senk *et al*, 2017). Overexpression of NADH oxidase in both *E. coli* (Vemuri *et al*, 2006) and *S. cerevisiae* (Vemuri *et al*, 2007) reduced the NADH/NAD⁺ ratio and suppressed overflow metabolism. One key difference between *P. aeruginosa* rhamnolipid production and the overflow metabolism of other systems is that rhamnolipids are controlled by quorum-sensing in addition to nutrient cues (Boyle *et al*, 2015), which results in a cell-density-dependence.

Should rhamnolipid production provide a fitness benefit for *P. aeruginosa* under any environmental conditions? We have previously shown that rhamnolipid production is dispensable for normal growth of *P. aeruginosa* in laboratory conditions: the $\Delta rhIA$ mutant is an engineered rhamnolipid non-producer defector but has almost the same growth rate (van Ditmarsch & Xavier, 2011) and very similar metabolomic profiles (Boyle *et al*, 2017) as the wild-type. This is consistent with our flux-balance analysis of *Pseudomonas* metabolism, where shutting down rhamnolipid secretion did not impact the growth rate because the excess carbon could be alternatively secreted in other forms such as acetate (Fig. 5). Notably, $\Delta rhIA$ had higher intracellular levels of gamma-Glutamylcysteine (Supplementary Fig. 7), the

immediate precursor of glutathione. Since glutathione is a well-known antioxidant that protects cells from ROS damage (Ezraty *et al*, 2017), $\Delta rhIA$ may exhibit slight oxidative burden. This could explain why the $\Delta rhIA$ has a marginally lower fitness and fails to invade the wild-type in swarming competitions (de Vargas Roditi *et al*, 2013; Monaco *et al*, 2020). Our mechanism proposes that increased oxidative burden may be related to altered redox ratio (NAD(P)H/NAD(P)⁺), considering that fatty acid biosynthesis that provides precursors for rhamnolipid production regenerates NAD(P)⁺ from NAD(P)H. Notably, the active shutdown of rhamnolipid production in redox imbalanced condition would save precious carbon and enzyme resources to respond to oxidative stress.

Our results confirm the role that metabolic byproducts play in linking single-cell physiology and social behavior. Microorganisms secrete various products, including iron scavenging siderophores (Griffin *et al*, 2004), extracellular enzymes (Altindis *et al*, 2015), toxins and cell-cell signaling molecules (Schmidt *et al*, 2019). In synthetic and natural microbial communities the products of one microorganism can impact another, cooperatively or competitively (Xavier, 2011). Our model proposes that the choice of a given molecule to drive a social interaction is not arbitrary: molecules that conciliate individual-level and population-level interests are more likely to drive microbial sociobiology.

Materials and Methods

Media. Glycerol and succinate synthetic media were prepared with 800 ml of Milipore water, 200 ml of 5X minimal salts buffer, 1ml of 1 M magnesium sulphate, 0.1 ml of calcium sulphate with 0.5 gN/l nitrogen (ammonium sulphate), iron at 5 μ M (iron III sulphate) and 3.0 gC/l glycerol or succinate, respectively. Swarming agar consisted in 800ml of Milipore water, 200 ml of 5X minimal salts buffer, 1ml of 1 M magnesium sulphate, 0.1 ml of calcium sulphate, 25 ml of 200 g/l solution of casamino acids (Bacto TM from BD, Sparks,MD) with

0.5% of agar. 5X stock minimal salts buffer was prepared with 12 g of Na₂HPO₄ (anhydrous), 15 g of KH₂PO₄ (anhydrous) and 2.5 g of NaCl into 1 l water.

Succinate and glycerol growth curve assays. The clinical isolates were inoculated in 3 mL of lysogen broth (LB) and incubated at 37°C overnight with shaking at 250 rpm. 500 µL of cell culture was pelleted and washed 3 times with PBS. 0.0025 OD₆₀₀ units were inoculated into 3.0 gC/l glycerol or succinate minimal medium in BD Falcon (BD Biosciences, San Jose, CA) 96 well flat-bottom plates, with 150 µL of suspension per well. The plate was incubated during 48 hours at 37°C in a Tecan Infinite M1000 or Tecan Infinite M1000 Pro plate reader (Männedorf, Switzerland), with an orbital shaking of 4 mm of amplitude. OD₆₀₀ was measured in 10 minutes intervals.

Swarming assay Swarming assays were performed as described previously (Xavier *et al*, 2011; van Ditmarsch *et al*, 2013). The clinical isolates were inoculated in 3 mL of LB and incubated at 37°C overnight, with shaking. 500 µL of the culture was pelleted and washed twice with PBS. 2 µL of this suspension was spotted on the surface of swarming agar without penetrating the agar with the pipette tip. Swarming medium contains 0.5% agar (Bacto) and is supplemented with 5g/L casamino acids, 1mM MgSO₄, 0.1mM CaCl₂ and 1X salt buffer (12g/L Na₂HPO₄ (Fisher Scientific), 15g/L KH₂PO₄ (Fisher Scientific) and 2.5g/L NaCl, pH6.7). The plates were incubated at 37°C ~24 hours. Two replicates were done per strain. In each batch of swarming assays PA14 was used as control.

Rhamnolipid production quantification. The production of rhamnolipids was assessed by drop-collapse assay (Jain & Collins; Chen *et al*, 2007). We placed 50 µL of the culture's supernatant on a polystyrene surface (the lid of a 96 well plate). The presence of rhamnolipids decreases the surface tension of the liquid, making the drop collapse. We considered a strain rhamnolipid non-producer if the drop does not spread, a mild-producer if

spreads, but not enough to pass over the lid's circle that correspond to the wells, and producer if spreads over it.

Image analysis and quantification of swarming phenotype. Images of the swarming plates were obtained with a Chemidoc gel doc imager (Bio Rad). The extraction of the morphological features from the images of the swarming plates were analyzed using Matlab bwmorph function. The features extracted from the images were perimeter of the colony, maximum length (the longitude of the rectangle that fits the colony), area percentage of the plate occupied by the colony, circularity (measured as $4\pi/P^2$, where P is the perimeter of the colony), longitude of the skeleton (skeletonization, consisting in all the points that are at equal distance to their nearby borders) and eccentricity (the eccentricity of the fitted ellipse). The feature values of each strain were normalized by the corresponding values of the PA14 strain in the same experiment. The swarming score (S_i) for each clinical isolate i is defined as a linear combination of the maximum length (L_i) and circularity (C_i) of its swarming colony (the two most discriminative features that separate strong swimmers from weak swimmers; see Supplementary Fig. 1)

$$S_i = 0.60(L_i - \tilde{L}) - 0.80(C_i - \tilde{C}) \quad (1)$$

where $\tilde{L}$ and $\tilde{C}$ are the mean values of L_i and C_i across all clinical isolates respectively, and the linear coefficients maximize the variance of the swarming score. To binarize the swarming phenotype, a strain is called swarmer if its swarming score is higher than the median swarming score across all strains (otherwise it is a non-swarmer).

Cross-strain reciprocal BLAST. To identify orthologous gene across all *P. aeruginosa* strains, systematic cross-strain reciprocal BLAST was performed. Protein sequences of each strain and the type strain PA14 were mutually queried against and the BLAST hits (genes)

with <80% coverage were removed. Two genes are orthologous if they were mutually the best hit to each other and genes (from other strains) that are orthologous to the same PA14 gene were considered orthologous to each other.

Phylogenetic analysis. The phylogenetic tree of clinical *P. aeruginosa* strains (Fig. 1A) was constructed from core genomes as previously described (Yan *et al*, 2017). Moran's I test was carried out using the ape package in R (Paradis & Schliep, 2019). The ancestral state of swarming and rhamnolipid production was reconstructed using corHMM package (Beaulieu *et al*, 2013).

Growth curve analysis. We first normalized the growth curves in different experiments (plates) by matching their corresponding controls (PA14). The plate-specific coefficients were obtained by solving a linear regression model 'log(OD of PA14) ~ Plate + Time' (the Wilkinson notation) using Matlab function fitlm, where Plate is plate ids and Time is time points (both are categorical variables). Then the growth curves in each plate were normalized by multiplying the adjustment coefficient of the plate. These normalized growth curves were then smoothed by Savitzky-Golay filter (sgolayfilt function in Matlab). The growth phases (phase I, II, III) were determined by the following rules: (phase I): first-order derivative >0 and second-order derivative >0; (phase II) first-order derivative >0 and second-order derivative <0; (phase III) first-order derivative <0. The mean and maximum specific growth rate for each growth phase were estimated from the best-fit model (highest R2) among three models of bacterial growth curves: (1) exponential model $y = y_0 + y_1 e^{\mu t}$; (2) Zwietering-Logistic model $y = y_0 + (A - y_0) \left(1 + e^{\frac{4\mu}{A}(\lambda - t) + 2}\right)^{-1}$; (3) Zwietering-Gompertz model $y = y_0 + (A - y_0) e^{-e^{\frac{\mu e}{A}(\lambda - t) + 1}}$, where $y_0, y_1, \mu, \lambda, A$ are the parameters to fit. Both non-negative factorization and random forest regression were performed in python, by sklearn.decomposition.NMF and sklearn.ensemble.RandomForestClassifier respectively.

Metabolite extraction. All *P. aeruginosa* strains were grown until $OD_{600} = 0.2$ (end of exponential phase of growth) in glycerol minimal media. Bacteria was then loaded into 0.25 μ m nylon membranes (Millipore) using vacuum, transferred to pre-warmed hard agar plates with the same medium composition and incubated at 37°C during 2.5 h. The filters were then passed to 35 mm polystyrene dishes (Falcon) with 1 mL of 2:2:1 methanol:acetonitrile:H₂O quenching buffer and incubated there during 15 minutes on dry ice. Cells were removed by scraping and the lysate containing quenching buffer was transferred to 1.5 mL tubes and centrifuged at 16000 rpm for 10 minutes at 4°C. Supernatant transferred to fresh tubes and stored at -80°C.

Metabolomic data preprocessing. The extracts were profiled using liquid-chromatography coupled to mass spectrometry (LC-MS), identifying a total of 92 compounds (Supplementary Fig. 5). Some compounds contained missing values. These missing values in metabolite abundance can be (1) truly missing; (2) present in a sample but its level is below detection limit; (3) present in a sample at a level above the detection limit but missing due to failure of algorithms in data processing. Here we assume that a metabolite with missing values in all three replicates is truly missing in the sample and removed from our analysis (Supplementary Fig. 5). However, if the missing values were only found in one or two replicates, the missing values were imputed by the average of the non-missing values. After that imputation all compounds with missing values were removed (Supplementary Fig. 5).

The peak areas were normalized using Cross-Contribution Compensating Multiple Standard Normalization (CCMN) (Redestig *et al*, 2009) with NormalizeMets R package (De Livera *et al*, 2018). This method relies on the use of multiple internal standards. Since LC-MS lacks such internal standards, we used instead a set of metabolites assumed to be constant across all the strains. They were selected with a Kuskal-Wallis test, adjusting the *p*-value with

Benjamini-Hochberg method. The ones with a p -value above 0.05 were considered constant (pyruvate, methylglyoxal, (S)-2-Acetolactate, Tyramine, D-Glucose, (S)-Lactate, N-acetyl-L-glutamate 5-semialdehyde, 4-Aminobutyraldehyde and Glycine), therefore after the normalization step they were removed (indicated in red, Fig. 3A).

Hierarchical clustering of metabolomic data. The hierarchical clustering of the normalized metabolomic data (Fig. 3B) was performed using gplots R package (Warnes *et al.* 2009), with Euclidean distance and Ward's aggregation method. The clustering was performed with all metabolites; however, 16 metabolites (indicated in red, Fig. 3A) with unknown compound identity were removed from Fig. 3B and downstream analyses. These unknown compounds are not artifacts arising from LC-MS instruments since their peaks were detected in all strains. Fumarate and guanosine, which were only putative initially, were bioinformatically confirmed as all other compounds with the same molecular weight are produced by enzymatic reactions missing in our clinical isolates.

Metabolic pathway enrichment. The differential metabolites between rhamnolipid producers and non-producers was determined by a Mann-Whitney test, with p -values adjusted with Benjamini-Hochberg method and a significance level of 0.05. These compounds were fed to FELLA algorithm (Picart-Armada *et al.*, 2018, 2017). FELLA retrieves a graph describing the relationships among pathway, module, enzyme, reaction and compounds of *Pseudomonas aeruginosa* strain UCBPP-PA14 from the KEGG database. The graph was then used as an input network of differential compounds for its diffusion algorithms (Vandin *et al.* 2011). The output of FELLA consists of all subnetworks of the entries predicted to have a high connectivity with the differential compounds. We filtered the subnetwork entries to keep only metabolic pathways.

OPLS-DA model. OPLS-DA model of metabolomics data was built using ropls R package (Thévenot *et al*, 2015), fixing the number of orthogonal components to 3. R^2 and Q^2 , key parameters for assessing the validity of the model, were assessed with 7-fold cross validation. The significance of the model was determined by permutation test ($n = 2000$). The p -value corresponds to the proportion of Q^2_{perm} above Q^2 . With a p -value below 0.05 we considered the model significant. The loadings of the predictive component of the model were extracted to determine how each metabolite contributes to the separation according to the phenotype.

Genome-scale modeling. Custom Python codes were developed with the COBRApy package (Ebrahim *et al*, 2013) to carry out all metabolic flux modeling and simulations in the paper. Since iJN1411 model was developed for *Pseudomonas putida*, we removed genes and associated reactions that are missing in all our strains but present in the iJN1411 model. The futile cycles involving NADH, NADPH, and GSH were also removed. The modified iJN1411 model was further expanded by adding rhamnolipid biosynthesis pathway involving 9 new metabolites and 12 new reactions. The metabolites, reactions and genes of the final model are given in JSON format (Supplementary File 4).

The boundary fluxes of the model were set to mimic the composition of the glycerol minimum medium. For C:N=10, the lower bounds of glycerol and ammonium fluxes were set to -10 and -3 respectively. For C:N=3, the lower bounds were set to -3 and -3 respectively. The flux unit is mmol/gDW/h throughout the paper. To constrain the total producing flux of NADH (the same for NADPH and GSH) at a certain value C , we first defined a binary variable X_k for each NADH-involving reaction k to indicate whether NADH is produced by this reaction. Given the stoichiometric coefficient of NADH in this reaction (s_k) and its flux value (f_k), the mathematical constraints for X_k was set by $X_k = 1$ for $s_k f_k > 0$ and $X_k = 0$ otherwise. Therefore, the constraint that equalizes the total NADH producing flux and a

constant C is simply $\sum_k s_k X_k f_k = C$. However, both X_k and f_k are variables and such quadratic constraint has not yet been supported by COBRApy. We overcame this difficulty by defining $A_k = X_k f_k$ and linearized the product with the following two inequalities: $X_k l_b \leq A_k \leq X_k u_b$ and $f_k - (1 - X_k)u_b \leq A_k \leq f_k - (1 - X_k)l_b$, where l_b and u_b are the lower and upper bounds of f_k . The two constraints ensure that $A_k = 0$ when $X_k = 0$ and $A_k = f_k$ when $X_k = 1$. The minimum/maximum flux values of byproduct secretion were simulated by flux variability analysis at maximum growth rate.

Detection of hydrogen peroxide (H_2O_2). The H_2O_2 level in the extracellular medium was quantified with Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Carlsbad, USA, Catalog no. A22188). 500 μ L of cell suspension were spinned down after overnight growth in LB medium, washed twice in PBS and normalized to OD 1. Each reaction was done in a final volume of 100 μ L, with a final concentration of 50 μ M of Amplex® Red reagent, 0.1 U/mL of HRP (Horseradish Peroxidase) and 0.2 cell OD, in glycerol synthetic medium, in BD Falcon (BD Biosciences, San Jose, CA) 96 well flat-bottom plates. The first column of the plate corresponded to the reaction without cells (the volume was substituted by PBS), and the last column instead of cells contained H_2O_2 (final concentration 10 μ M). OD₆₀₀ and fluorescence 530/590 nm was measured in 10 minutes intervals (48 h 37°C).

Hydrogen peroxide (H_2O_2) degradation. H_2O_2 was removed from environment by cells which degrade H_2O_2 intracellularly. The cumulative H_2O_2 removal curve was calculated by subtracting the values of emission of the wells containing each strain from the values of emission of the wells without cells or H_2O_2 . Then they were normalized to the relative scale by dividing their values by the corresponding values (at the same time points) of the control strain (PA14) in the same experiment (plate). These normalized curves were then smoothed

by the Savitzky-Golay filter (sgolayfilt function in Matlab). The H₂O₂ removal rate per cell was calculated by dividing the first-order derivative of the cumulative removal curves by OD values. To estimate the effect of rhamnolipid production on per-cell H₂O₂ removal rate, we developed a linear mixed-effect model that extends the simple linear regression model by considering both fixed and random effects. The model was specified by the Wilkinson notation ‘RemovalRate ~ Time + RHL + (1|CurveID)’, where RemovalRate is per-cell H₂O₂ removal rate, Time is time point (categorical variable), RHL is categorical rhamnolipid production (non-producer, mild-producer, and strong-producer), and CurveID is the unique ID for each strain and replicate. The expression ‘(1|CurveID)’ indicates that CurveID was modeled as random effects. The model was solved by fitlme in Matlab.

Data availability

All experimental and simulation data that support the conclusions of this study are available from the main text and Supplementary Information. The source codes for generating the figures of this study are available from https://github.com/liaochen1988/Source_code_for_Pseudomonas_Metabolomics_Paper.

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 861
 862

Table 1 Genes that are missing in non-rhamnolipid-producers but present in all producers

Non-producers	Missing genes
F5677	<i>arsB, arsC, arsH, arsR, chiC, dacB, fliM, gcd, qscR, gntK, gntR,</i>
F63912	<i>lasR</i>
H27930	<i>arnA, pelA, pcaB, pcaD, pfeA, icmF3, rrsM, pcd, pirR, pctA,</i>
M1608	<i>tauD, opdG, pdxA, hcnC, hcnB, hcnA, glgA, ybhO, glgB, katE, glgP, ligD, ada, opdO, kynB, fusA2, cynR, cynT, cynS, cmrA, gor, galU, amrB, gnyD, gnyB, gnyH, gnyA, gnyL, hmgA, fahA, maiA,</i>
M55212	<i>crc, rph</i>
S86968	<i>kdsB, cysP, hcnC, hcnB, hcnA, pdxA, opdG, nfxB, cupB3</i>
W36662	<i>rhlR, rhlI, cupC1, pilC, pilS</i>
W60856	<i>lasR, pirA</i>

Figures

Figure 1. Diversity of swarming across the *P. aeruginosa* phylogeny of core genomes. **A.** Phylogeny of clinical isolates obtained from patients with cancer at MSKCC (Yan *et al*, 2017, 2019) together with reference type strains PAO1, PA14 and PA7. The tissue where each isolate was originally isolated is labeled by circle colors. **B.** Swarming and rhamnolipid production phenotypes. The swarming ability of these strains is represented by the actual swarming images on the agar plates (left column). The ability of producing rhamnolipids of these strains is semi-quantitatively indicated by circle sizes (right column). Both phenotypes do not correlate with phylogeny. All swimmers can

secrete rhamnolipids but some rhamnolipid producers cannot swarm. **C.** Growth curves in a synthetic minimal medium using glycerol as the sole carbon source. Strain M6075 was excluded from phylogenetic analysis due to the previous failure in sequencing and assembling its genome.

Figure 2. Growth curve features distinguish rhamnolipid producers from non-producers. **A,B.** Unsupervised feature selection using non-negative matrix factorization (NNMF), which decomposes growth curves of all *Pseudomonas* isolates into three additive basis functions (features) such that each growth curve can be approximately represented by the weighted sum of these functions. **A.** Growth curves from both rhamnolipid producers (orange) and non-producers (blue). **B.** Decomposed components (basis function multiplied by weights; left panels) and weights (right panels) from NNMF grouped by rhamnolipid (RL) production. The shaded areas represent 95% bootstrap confidence interval of the mean. **C-E.** Supervised feature selection using Random Forest classifier. **C,D.** Feature extraction method. Each growth curve (excluding the initial lag phase) was divided into three phases (C) and each phase was described by 7 quantitative features (D). **E.** Ranking of feature importance in

Figure 3. Variance and clustering analysis of metabolomics across *Pseudomonas aeruginosa* strains. **A.** Logarithm of raw peak area of all metabolites identified by LC-MS (Liquid chromatography-Mass spectrometry). The metabolites in red were used as internal standard in the data normalization step but removed from the dataset afterwards. **B.** Hierarchical clustering of the metabolic profiles using Euclidean distance and Ward aggregation method. Each row represents one metabolite and each column represents a specific sample (both strain and sample number are indicated).

Figure 4. Comparative metabolomics between rhamnolipid producers (include both mild and strong production levels) and non-producers. **A.** The scores plot of the OPLS-DA (orthogonal partial least squares discriminant analysis) model. The first principle component separated the rhamnolipid non-producers from producers. **B.** OPLS-DA loading values for the first principle component of a selected number of metabolites. Metabolites with asterisk (*) are significantly different between producers and non-producers (adjusted p-value of a Mann-Whitney test < 0.05). **C.** Differential abundance of metabolites involved in reactions catalyzed by some Fe-S-containing enzymes whose activities are inhibited by reactive oxygen species (ROS). Abbreviations: *acnA*

(aconitate hydratase A), *acnB* (aconitate hydratase B), *sdhB* (succinate dehydrogenase subunit), *fumA* (fumarase A), *fumB* (fumarase B), *gltB* (glutamate synthase subunit), *gltD* (glutamate synthase subunit).

Figure 5 Growth and rhamnolipid secretion are constrained by redox status. The redox stress levels are perturbed by altering fluxes of NADH (A. reduced nicotinamide adenine dinucleotide) NADPH (B. reduced nicotinamide adenine dinucleotide phosphate) and GSH (C. reduced glutathione). Upper panels are predicted maximum growth rates and lower panels are predicted maximum byproduct secretion fluxes. C:N indicates the carbon-to-nitrogen ratio between glycerol and ammonium in the culture medium. C:N=3 and C:N=10 represent carbon- and nitrogen-limiting conditions respectively. Abbreviations: HAA: 3-(3-hydroxyalkanoyloxy) alkanoate; monoRL: monorhamnolipid; diRL: dirhamnolipid; αKG: alpha-ketoglutarate.

Figure 6. Comparison of the ability of removing culture medium hydrogen peroxide (H_2O_2) among strong rhamnolipid producers (+), weak producers (+/-), and non-producers (-). **A.** Population density. **B.** The total amount of hydrogen peroxide removed from the environment. Negative values indicate net cellular production of hydrogen peroxide released to the environment. **C.** The specific hydrogen peroxide removal rate. In both **B** and **C**, each trajectory of H_2O_2 fluorescence intensity was normalized to the averaged trajectory of the wild-type UCBPP-PA14 strain.

Figure 7. Schematic model showing how metabolism regulates evolution of swarming and rhamnolipid production in *P. aeruginosa*. During aerobic growth, cells rely on the TCA cycle and electron transport chain for central energy metabolism, which generates ROS (reactive oxygen species) as byproduct. Particularly, the membrane-located succinate dehydrogenase (SDH), which oxidizes succinate (succ in short, red dot, lower in rhamnolipid producers) to fumarate (fum in short, blue dot, higher in rhamnolipid producers), is an important site of ROS production and serves as the bottleneck of TCA cycle when ROS is excessive. **A.** Rhamnolipid producers are able to effectively remove ROS; therefore, their TCA cycle fluxes are only minimally inhibited by ROS and remain high to support fast growth. When cells reach certain density and nitrogen becomes limited, rhamnolipid production is turned on by quorum-sensing. **B.** Rhamnolipid non-producers ineffectively remove ROS and excessive ROS inhibits SDH, resulting in higher level of succinate and lower level of fumarate compared to rhamnolipid producers. The ROS-mediated inhibition significantly slows down the TCA

944 cycle and cell growth. The increased ROS level is thought to impair quorum-sensing in non-producers
 945 and subsequently abolishes rhamnolipid production under the same culture medium of producers. Our
 946 model further suggests that null mutations and entire loss of genes that play important roles in redox
 947 stress sensing, signaling and protection drive the observed diversity of swarming and rhamnolipid
 948 production phenotypes along evolution.

949 Supplementary Figures

950

951

952 **Supplementary Figure 1.** Principle component analysis and biplot of the swarming phenotype. Six
 953 morphological features were extracted from quantitative imaging analysis (see **Materials and**
 954 **Methods** in the main text), including maximum length (MaxLength), skeleton, perimeter, area of
 955 swarming colony (AreaPercentage), circularity and eccentricity. The MaxLength and circularity were
 956 the top two features that separate strong swimmers (bold font-weight) from weak swimmers (normal
 957 font-weight). The top 3 strains that have visually the largest (best swimmers) and smallest (worst
 958 swimmers) swarming colonies are highlighted in red and blue respectively with their swarming images

959 shown alongside. The abilities of these strains to produce rhamnolipids are also indicated by triangles
960 (producers) and dots (non-producers).

961
962 **Supplementary Figure 2.** Phylogenetic ancestor state reconstruction of rhamnolipid production (A)
963 and swarming phenotypes (B). For both (A) and (B), pie charts at the ancestor nodes of branches
964 represent relative likelihood proportion of each possible phenotypic state. We rooted the tree with
965 PA7, a *P. aeruginosa* isolate that is often used as an outlier to root phylogenetic trees. RL,
966 rhamnolipid production.

967

968 **Supplementary Figure 3.** Clustergram of growth curves of *P. aeruginosa* clinical isolates and three
 969 type strains PA14, PAO1 and PA7 in glycerol minimal medium. Euclidean distance was used as the
 970 measure of similarity. The strains that are able to swarm are indicated in bold. Most of the swarmers
 971 are clustered together. The swarmers that do not fall into this cluster include F30658 and PAO1, both
 972 of which are mild swarmers, as well as F23197 whose growth curve has a relatively longer lag time.

Supplementary Figure 4. Growth curve of *P. aeruginosa* strains in glycerol minimal medium. Phase I, II, and III are colored by red, blue and green respectively.

Supplementary Figure S5. A. The same as Fig. 3A of the main text but additionally includes metabolites with putative molecular identifications. These putative metabolites were initially included in clustering metabolomics but later removed from the heatmap (Fig. 3B of the main text). **B.** Metabolite peak area before (left panel) and after (right panel) imputation. The missing value proportion of a metabolite in the x-axis represents the frequency of missing values of the metabolite across all replicates of all strains in our study. The peak range of a metabolite in the y-axis is defined as the maximum peak area minus the minimum peak area among all non-missing values of the same metabolite across all our strains.

Supplementary Figure 6. The loading values of all predictive metabolites of the OPLS-DA model. The differential metabolites between producers and non-producers were determined by a Mann Whitney test (adjusted p -values with Benjamini-Hochberg method) with a level of significance of

991 0.05 (bars with black outline) and used as input for a metabolic pathway enrichment with FELLA
992 algorithm. The colors indicate the mapped metabolite pathway for each metabolite.

993

994 **Supplementary Figure 7.** Volcano plot of metabolomics data between wild-type *P. aeruginosa*
 995 UCBPP-PA14 strain and its *rhlA* mutant grown in glycerol minimal medium (replotted with
 996 permission from (Boyle *et al*, 2017)).

Supplementary Figure 8. Theoretical estimation of threshold carbon (glycerol):nitrogen (ammonium) ratio above which carbon is in excess in the sense that carbon release through rhamnolipids and central carbon metabolites does not compromise biomass production. Abbreviations: HAA: 3-(3-hydroxyalkanoyloxy)alkanoate; monoRL: monorhamnolipid; diRL: dirhamnolipid; αKG: alpha-ketoglutarate.

Supplementary Figure 9. Growth curve of *Pseudomonas aeruginosa* strains in succinate minimal medium. Phase I, II, and III are colored by red, blue and green respectively.

Supplementary Figure 10. Comparison of growth curve features of *Pseudomonas aeruginosa* strains grown in glycerol and succinate minimal medium.

Supplementary Data

Supplementary Table 1: output of the FELLA algorithm. A set of differential compounds was determined with a Mann-Whitney U test between rhamnolipid producers and rhamnolipid non producers (p -value adjusted with Benjamini-Hochberg method, $\alpha = 0.05$). These compounds were used for applying diffusion algorithms over a graph consisting in all entries for *Pseudomonas aeruginosa* strain UCBPP-PA14 in KEGG database. The entries shown in the table are the ones with a significative probability of receiving part of the simulated flux.

KEGG.id	Entry.type	KEGG.name	p.score
pau00020	pathway	Citrate cycle (TCA cycle) - Pseudomonas aerug...	2.051E-02
		Pentose phosphate pathway - Pseudomonas	
pau00030	pathway	aerug...	2.657E-04
pau00250	pathway	Alanine, aspartate and glutamate metabolism -...	3.502E-02
		Cysteine and methionine metabolism -	
pau00270	pathway	Pseudomo...	3.106E-04
pau00290	pathway	Valine, leucine and isoleucine biosynthesis -...	1.000E-06
pau00620	pathway	Pyruvate metabolism - Pseudomonas aeruginosa ...	3.766E-02
pau00630	pathway	Glyoxylate and dicarboxylate metabolism - Pse...	1.105E-02
pau00660	pathway	C5-Branched dibasic acid metabolism - Pseudom...	8.242E-03
pau01503	pathway	Cationic antimicrobial peptide (CAMP) resista...	3.891E-02
pau02060	pathway	Phosphotransferase system (PTS) - Pseudomonas...	4.222E-02
M00001	module	Glycolysis (Embden-Meyerhof pathway), glucose...	1.613E-02
M00002	module	Glycolysis, core module involving three-carbo...	4.022E-03
M00003	module	Gluconeogenesis, oxaloacetate => fructose-6P	2.299E-03
		Pentose phosphate pathway (Pentose phosphate	
M00004	module	...	3.993E-03

M00005	module	PRPP biosynthesis, ribose 5P => PRPP	1.000E-06
M00007	module	Pentose phosphate pathway, non-oxidative phas...	1.030E-04
M00009	module	Citrate cycle (TCA cycle, Krebs cycle)	7.517E-04
M00010	module	Citrate cycle, first carbon oxidation, oxaloa...	4.789E-04
M00011	module	Citrate cycle, second carbon oxidation, 2-oxo...	8.189E-03
M00012	module	Glyoxylate cycle	1.577E-03
M00019	module	Valine/isoleucine biosynthesis, pyruvate => v...	5.143E-03
M00032	module	Lysine degradation, lysine => saccharopine =>...	2.928E-04
M00049	module	Adenine ribonucleotide biosynthesis, IMP => A...	3.245E-02
M00149	module	Succinate dehydrogenase, prokaryotes	4.051E-02
M00165	module	Reductive pentose phosphate cycle (Calvin cyc...	8.362E-04
M00167	module	Reductive pentose phosphate cycle, glyceralde...	2.917E-04
M00173	module	Reductive citrate cycle (Arnon-Buchanan cycle...	1.377E+09
M00308	module	Semi-phosphorylative Entner-Doudoroff pathway...	2.511E-02
M00344	module	Formaldehyde assimilation, xylulose monophosp...	5.467E-03
M00374	module	Dicarboxylate-hydroxybutyrate cycle	2.552E-02
M00432	module	Leucine biosynthesis, 2-oxoisovalerate => 2-o...	1.000E-06
M00535	module	Isoleucine biosynthesis, pyruvate => 2-oxobut...	1.000E-06
M00570	module	Isoleucine biosynthesis, threonine => 2-oxobu...	8.290E-04
M00620	module	Incomplete reductive citrate cycle, acetyl-Co...	1.000E-06
M00633	module	Semi-phosphorylative Entner-Doudoroff pathway...	3.830E-02
M00740	module	Methylaspartate cycle	4.398E-02
1.1.1.85	enzyme	3-isopropylmalate dehydrogenase	1.000E-06
		glyceraldehyde-3-phosphate dehydrogenase	
1.2.1.12	enzyme	(pho...	1.578E-03

		glyceraldehyde-3-phosphate dehydrogenase	
1.2.1.9	enzyme	(NAD...	4.815E-04
1.3.5.1	enzyme	succinate dehydrogenase	5.028E+08
1.3.5.4	enzyme	fumarate reductase (quinol)	5.028E+08
1.4.1.9	enzyme	leucine dehydrogenase	1.032E-02
1.8.4.15	enzyme	protein dithiol oxidoreductase (disulfide-for...	3.891E-02
2.2.1.1	enzyme	transketolase	1.838E+08
2.2.1.2	enzyme	transaldolase	5.939E-04
2.3.3.1	enzyme	citrate (Si)-synthase	1.000E-06
2.3.3.13	enzyme	2-isopropylmalate synthase	1.000E-06
2.4.2.1	enzyme	purine-nucleoside phosphorylase	1.000E-06
2.4.2.2	enzyme	pyrimidine-nucleoside phosphorylase	1.000E-06
2.5.1.49	enzyme	O-acetylhomoserine aminocarboxypropyltransfer...	4.137E-02
2.6.1.2	enzyme	alanine transaminase	1.000E-06
2.6.1.42	enzyme	branched-chain-amino-acid transaminase	1.985E-02
2.6.1.66	enzyme	valine---pyruvate transaminase	1.000E-06
2.7.1.15	enzyme	ribokinase	1.791E-02
2.7.1.221	enzyme	N-acetylmuramate 1-kinase	1.000E-06
2.7.2.3	enzyme	phosphoglycerate kinase	1.747E-02
2.7.6.1	enzyme	ribose-phosphate diphosphokinase	1.000E-06
2.7.9.2	enzyme	pyruvate, water dikinase	7.185E-03
2.8.3.18	enzyme	succinyl-CoA:acetate CoA-transferase	1.672E-02
3.1.3.105	enzyme	N-acetyl-D-muramate 6-phosphate phosphatase	1.000E-06
3.1.3.11	enzyme	fructose-bisphosphatase	3.302E-02
3.1.3.48	enzyme	protein-tyrosine-phosphatase	1.524E-02

3.2.2.10	enzyme	pyrimidine-5'-nucleotide nucleosidase	1.000E-06
3.2.2.4	enzyme	AMP nucleosidase	1.000E-06
3.4.21.107	enzyme	peptidase Do	1.929E-02
3.4.24.40	enzyme	serralysin	3.891E-02
3.5.1.28	enzyme	N-acetylmuramoyl-L-alanine amidase	1.000E-06
3.5.4.2	enzyme	adenine deaminase	1.786E-02
3.6.1.13	enzyme	ADP-ribose diphosphatase	7.396E+08
3.7.1.20	enzyme	3-fumarylpyruvate hydrolase	2.495E-02
4.1.1.31	enzyme	phosphoenolpyruvate carboxylase	4.009E-02
4.1.2.13	enzyme	fructose-bisphosphate aldolase	2.966E-03
4.1.2.14	enzyme	2-dehydro-3-deoxy-phosphogluconate aldolase	2.363E-02
4.1.2.48	enzyme	low-specificity L-threonine aldolase	9.416E-04
4.1.3.1	enzyme	isocitrate lyase	1.814E-02
4.2.1.2	enzyme	fumarate hydratase	1.000E-06
4.2.1.20	enzyme	tryptophan synthase	3.153E-02
4.2.1.3	enzyme	aconitate hydratase	1.000E-06
4.2.1.33	enzyme	3-isopropylmalate dehydratase	1.000E-06
4.2.1.35	enzyme	(R)-2-methylmalate dehydratase	1.000E-06
4.2.3.1	enzyme	threonine synthase	3.155E-02
4.3.1.1	enzyme	aspartate ammonia-lyase	1.000E-06
4.3.1.19	enzyme	threonine ammonia-lyase	1.000E-06
4.3.2.1	enzyme	argininosuccinate lyase	1.647E-02
4.3.2.2	enzyme	adenylosuccinate lyase	9.353E-04
5.1.3.1	enzyme	ribulose-phosphate 3-epimerase	1.624E-02
5.2.1.8	enzyme	peptidylprolyl isomerase	3.891E-02

5.3.1.1	enzyme	triose-phosphate isomerase	1.698E+08
5.3.1.6	enzyme	ribose-5-phosphate isomerase	2.338E-02
5.4.2.2	enzyme	phosphoglucomutase (alpha-D-glucose-1,6-bisph...	4.898E+09
6.2.1.5	enzyme	succinate---CoA ligase (ADP-forming)	6.932E-03
6.3.1.2	enzyme	glutamine synthetase	3.639E-02
6.4.1.1	enzyme	pyruvate carboxylase	4.182E+07
R00086	reaction	ATP phosphohydrolase	3.936E-02
R00122	reaction	ADP phosphohydrolase	3.029E-02
R00177	reaction	ATP:L-methionine S-adenosyltransferase	1.894E-02
R00182	reaction	AMP phosphoribohydrolase	1.000E-06
R00220	reaction	L-serine ammonia-lyase	2.595E-02
R00258	reaction	L-Alanine:2-oxoglutarate aminotransferase	4.726E-02
R00351	reaction	acetyl-CoA:oxaloacetate C-acetyltransferase (...)	1.000E-06
R00352	reaction	acetyl-CoA:oxaloacetate C-acetyltransferase [...]	1.000E-06
R00402	reaction	succinate:NAD+ oxidoreductase	1.000E-06
R00405	reaction	Succinate:CoA ligase (ADP-forming)	4.397E+09
R00432	reaction	Succinate:CoA ligase (GDP-forming)	2.166E-02
R00446	reaction	L-lysine:NAD+ 6-oxidoreductase (deaminating)	1.000E-06
R00479	reaction	isocitrate glyoxylate-lyase (succinate-formin...	3.522E-02
R00490	reaction	L-aspartate ammonia-lyase (fumarate-forming)	1.000E-06
R00510	reaction	cytidine-5'-monophosphate phosphoribohydrolas...	1.000E-06
R00653	reaction	N-formyl-L-methionine amidohydrolase	1.000E-06
R00751	reaction	L-threonine acetaldehyde-lyase (glycine-formi...	1.000E-06
R00782	reaction	L-cysteine hydrogen-sulfide-lyase (deaminatin...	1.000E-06
R00891	reaction	L-serine hydro-lyase (adding hydrogen sulfide...	1.000E-06

R00892	reaction	L-cysteine:NAD+ oxidoreductase	1.384E-02
R00893	reaction	L-Cysteine:oxygen oxidoreductase	7.899E-03
R00894	reaction	L-glutamate:L-cysteine gamma-ligase (ADP-form...	3.659E-03
R00895	reaction	L-Cysteine:2-oxoglutarate aminotransferase	1.786E-02
R00897	reaction	O3-acetyl-L-serine:hydrogen-sulfide 2-amino-2...	4.683E-03
R00901	reaction	L-cysteine hydrogen-sulfide-lyase (adding sul...	4.539E-03
R00994	reaction	(2R,3S)-3-methylmalate:NAD+ oxidoreductase	1.000E-06
R00996	reaction	L-threonine ammonia-lyase (2-oxobutanoate-for...	1.000E-06
R00999	reaction	O-Succinyl-L-homoserine succinate-lyase (deam...	2.925E-02
R01001	reaction	L-cystathionine cysteine-lyase (deaminating	3.769E-04
R01015	reaction	D-glyceraldehyde-3-phosphate aldose-ketose-is...	1.000E-06
R01049	reaction	ATP:D-ribose-5-phosphate diphosphotransferase	1.000E-06
R01051	reaction	ATP:D-ribose 5-phosphotransferase	1.000E-06
R01053	reaction	Ribose-5-phosphate:ammonia ligase (ADP-formin...	1.804E+07
R01054	reaction	ADP-ribose ribophosphohydrolase	9.884E+08
R01055	reaction	uracil hydro-lyase (adding D-ribose 5-phospha...	2.245E-03
R01056	reaction	D-ribose-5-phosphate aldose-ketose-isomerase	1.000E-06
R01057	reaction	D-Ribose 1,5-phosphomutase	1.000E-06
R01058	reaction	D-glyceraldehyde 3-phosphate:NADP+ oxidoreduc...	1.000E-06
R01059	reaction	ATP:D-glyceraldehyde 3-phosphotransferase	1.000E-06
R01061	reaction	D-glyceraldehyde-3-phosphate:NADP+ oxidoreduct...	1.357E-04
R01063	reaction	D-glyceraldehyde-3-phosphate:NADP+ oxidoreduc...	1.690E-04

R01066	reaction	2-deoxy-D-ribose-5-phosphate acetaldehyde-lyase...	1.494E-04
R01067	reaction	D-Fructose 6-phosphate:D-glyceraldehyde-3-ph...	5.048E+08
R01068	reaction	D-fructose-1,6-bisphosphate D-glyceraldehyde-...	8.170E+09
R01069	reaction	D-tagatose 1,6-bisphosphate D-glyceraldehyde-...	4.029E-04
R01070	reaction	beta-D-fructose-1,6-bisphosphate D-glyceralde...	8.852E+08
R01082	reaction	(S)-malate hydro-lyase (fumarate-forming)	1.000E-06
R01083	reaction	N6-(1,2-dicarboxyethyl)AMP AMP-lyase (fumarat...	2.469E-03
R01085	reaction	3-fumarylpyruvate fumarylhydrolase	1.563E-02
R01086	reaction	2-(Nomega-L-arginino)succinate arginine-lyase...	5.126E-03
R01087	reaction	Maleate cis-trans-isomerase	2.433E+09
R01113	reaction	Glutathione:L-cystine oxidoreductase	3.593E-02
R01128	reaction	5'-Inosinate phosphoribohydrolase	1.000E-06
R01213	reaction	acetyl-CoA:3-methyl-2-oxobutanoate C-acetyltr...	1.000E-06
R01215	reaction	L-Valine:pyruvate aminotransferase	3.535E-02
R01244	reaction	Adenine aminohydrolase	2.846E-02
R01270	reaction	Nicotinamide D-ribonucleotide phosphoribohydr...	1.000E-06
R01322	reaction	Citrate:CoA ligase (ADP-forming)	3.624E-04
R01324	reaction	citrate hydroxymutase	1.000E-06
R01325	reaction	citrate hydro-lyase (cis-aconitate-forming)	1.000E-06
R01364	reaction	4-fumarylacetoacetate fumarylhydrolase	2.477E-02
R01440	reaction	D-Xylulose-5-phosphate:formaldehyde glycolald...	4.322E+09
R01465	reaction	L-threonine:NAD+ oxidoreductase	1.000E-06
R01466	reaction	O-phospho-L-homoserine phosphate-lyase (addin...	6.537E+09
R01561	reaction	adenosine:phosphate alpha-D-ribosyltransferas...	2.961E-03
R01570	reaction	thymidine:phosphate deoxy-alpha-D-ribosyltran...	1.000E-06

R01621	reaction	D-xylulose 5-phosphate D-glyceraldehyde-3-phosph...	1.000E-06
R01641	reaction	sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosph...	1.000E-06
R01652	reaction	4-Methyl-2-oxopentanoate + CO2 <=> (2S)-2-Iso...	4.391E-03
R01827	reaction	sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosph...	1.594E-04
R01830	reaction	beta-D-Fructose 6-phosphate:D-glyceraldehyde-3-phosph...	3.088E+08
R01863	reaction	inosine:phosphate alpha-D-ribosyltransferase	7.366E-03
R01867	reaction	(S)-dihydroorotate:fumarate oxidoreductase	1.035E-03
R01876	reaction	uridine:phosphate alpha-D-ribosyltransferase	2.008E-03
R01969	reaction	Deoxyguanosine:orthophosphate ribosyltransferase	1.000E-06
R02147	reaction	guanosine:phosphate alpha-D-ribosyltransferase	1.837E-02
R02164	reaction	succinate:quinone oxidoreductase	1.000E-06
R02294	reaction	N-Ribosylnicotinamide:orthophosphate ribosyltransferase	4.332E-02
R02296	reaction	cytidine:orthophosphate alpha-D-ribosyltransferase	3.703E-02
R02297	reaction	Xanthosine:orthophosphate ribosyltransferase	4.035E-02
R02313	reaction	N6-(L-1,3-Dicarboxypropyl)-L-lysine:NAD+ oxidoreductase	1.000E-06
R02315	reaction	N6-(L-1,3-Dicarboxypropyl)-L-lysine:NADP+ oxidoreductase	1.000E-06
R02317	reaction	(S)-2-amino-6-oxohexanoate hydro-lyase (spontaneous)	1.000E-06
R02340	reaction	(1S,2R)-1-C-(indol-3-yl)glycerol 3-phosphate ...	7.357E-03
R02409	reaction	Coenzyme A:oxidized-glutathione oxidoreductase	4.474E-02
R02484	reaction	deoxyuridine:orthophosphate 2-deoxy-D-ribosyltransferase	1.000E-06
R02557	reaction	Deoxyadenosine:orthophosphate ribosyltransferase	1.000E-06
R02722	reaction	L-serine hydro-lyase [adding 1-C-(indol-3-yl)glycerol 3-phosphate ...]	3.011E-03
R02748	reaction	Deoxyinosine:orthophosphate ribosyltransferase	1.000E-06
R02749	reaction	2-deoxy-D-ribose 1-phosphate 1,5-phosphomutase	1.000E-06

L-2-aminoadipate-6-semialdehyde:NAD+ 6-			
R03102	reaction	oxidor...	1.000E-06
L-2-aminoadipate-6-semialdehyde:NADP+ 6-			
R03103	reaction	oxido...	1.000E-06
R03217	reaction	O-Acetyl-L-homoserine succinate-lyase (adding...	3.059E-02
R03260	reaction	O-Succinyl-L-homoserine succinate-lyase (addi...	2.438E-03
R03524	reaction	L-cysteine hydrogen-sulfide-lyase (adding HCN...	3.504E-02
R03896	reaction	(R)-2-Methylmalate hydro-lyase (2-methylmalea...	1.149E+08
R03898	reaction	2-methylmaleate hydratase	1.000E-06
R03968	reaction	2-Isopropylmalate hydro-lyase	1.000E-06
R04001	reaction	3-Isopropylmalate hydro-lyase	1.000E-06
L-2-aminoadipate-6-semialdehyde:NADP+ 6-			
R04390	reaction	oxido...	2.104E-02
R04426	reaction	(2R,3S)-3-isopropylmalate:NAD+ oxidoreductase	1.000E-06
R04559	reaction	1-(5'-Phosphoribosyl)-5-amino-4-(N-succinocar...	7.225E-03
R05598	reaction	Benzylsuccinate fumarate-lyase	1.083E-02
R05605	reaction	2-dehydro-3-deoxy-6-phospho-D-gluconate D-gly...	2.199E-03
R05636	reaction	1-Deoxy-D-xylulose-5-phosphate pyruvate-lyase...	3.417E-03
R06531	reaction	ATP:L-threonine O-phosphotransferase	2.361E-04
R06590	reaction	sedoheptulose-7-phosphate:D-glyceraldehyde-3-...	1.654E-02
R06789	reaction	(2Z,4E,7E)-2-Hydroxy-6-oxonona-2,4,7-triene-1...	1.163E-02
2-Methylnaphthalene + Fumarate <=> 2-			
R06903	reaction	Naphtylm...	2.194E-02
R06987	reaction	propanoyl-CoA:formate C-propanoyltransferase	2.218E-02
R07159	reaction	D-glyceraldehyde-3-phosphate:ferredoxin oxido...	1.000E-06

R07274	reaction	O-phospho-L-serine:hydrogen-sulfide 2-amino-2...	1.948E-04
R07399	reaction	Acetyl-CoA + Pyruvate + H2O <=> (R)-2-Methylm...	2.986E-03
R07456	reaction	D-ribulose 5-phosphate,D-glyceraldehyde 3-pho...	3.194E+09
R08323	reaction	7-Mercaptoheptanoic acid + L-Threonine + ATP ...	5.991E-03
R08555	reaction	(R)-lactate hydro-lyase (adding N-acetyl-D-gl...	9.758E-03
R08559	reaction	protein-N(pi)-phosphohistidine:N-acetylmurama...	1.000E-06
R10049	reaction	2-oxopropanal:D-fructose 1,6-bisphosphate gly...	4.546E-02
R10052	reaction	(2R,3S)-3-isopropylmalate:NAD+ oxidoreductase	1.000E-06
R10090	reaction	citrate:N6-acetyl-N6-hydroxy-L-lysine ligase ...	6.270E-03
R10170	reaction	(2R,3S)-3-isopropylmalate hydro-lyase (2-isop...	1.000E-06
R10270	reaction	(5R)-5-phosphooxy-L-lysine phosphate-lyase (d...	4.432E-04
R10343	reaction	succinyl-CoA:acetate CoA-transferase	1.608E-03
R10660	reaction	fumarate CoM:CoB oxidoreductase (succinate fo...	6.353E+09
R10677	reaction	citrate:L-glutamate ligase (ADP-forming)	2.358E-02
R10699	reaction	L-lysine:8-amino-7-oxononanoate aminotransfer...	1.201E-03
		D-glyceraldehyde-3-phosphate:NAD+	
R10860	reaction	oxidoreduct...	1.000E-06
R10888	reaction	C20914 + L-Threonine <=> C20915	1.339E-02
R10891	reaction	Tabtoxin + H2O <=> Tabtoxine-beta-lactam + ...	1.168E-03
R10892	reaction	tabtoxine-beta-lactam:L-threonine ligase (A...	8.832E-04
R10940	reaction	fumarate:L-2,3-diaminopropanoate ligase (AMP-...	6.289E-03
R10973	reaction	D-ribose-2,5-bisphosphate 2-phosphohydrolase	2.912E-03
R11024	reaction	ATP:N-acetyl-D-muramate 1-phosphotransferase	1.000E-06
		(S)-2-amino-6-oxohexanoate:NADP+	
R11679	reaction	oxidoreducta...	1.000E-06

		N-acetyl-D-muramate 6-phosphate	
R11785	reaction	phosphohydrol...	1.000E-06
R12308	reaction	L-2,3-diaminopropanoate:citrate ligase (2-[(L...	2.769E-03
R12353	reaction	D-Ornithine + Citrate + ATP <=> N5-Citryl-D-o...	4.337E-04
R12354	reaction	N5-Citryl-D-ornithine + Citrate + ATP <=> Sta...	3.695E-02
R12596	reaction	Spermidine + Citrate + ATP <=> N-Citryl-sperm...	2.685E-04
C00009	compound	Orthophosphate	9.902E-03
C00042	compound	Succinate	1.000E-06
C00058	compound	Formate	1.695E-02
C00073	compound	L-Methionine	3.505E-03
C00097	compound	L-Cysteine	1.000E-06
C00109	compound	2-Oxobutanoate	1.133E-02
C00117	compound	D-Ribose 5-phosphate	1.000E-06
C00118	compound	D-Glyceraldehyde 3-phosphate	1.000E-06
C00122	compound	Fumarate	1.000E-06
C00141	compound	3-Methyl-2-oxobutanoic acid	2.304E-02
C00158	compound	Citrate	1.000E-06
C00188	compound	L-Threonine	1.000E-06
C00672	compound	2-Deoxy-D-ribose 1-phosphate	1.000E-06
C00673	compound	2-Deoxy-D-ribose 5-phosphate	2.442E-02
C02226	compound	2-Methylmaleate	3.627E-04
C02504	compound	alpha-Isopropylmalate	1.000E-06
C02631	compound	2-Isopropylmaleate	1.000E-06
C02713	compound	N-Acetylmuramate	1.000E-06
C03145	compound	N-Formylmethionine	1.000E-06

C04076	compound	L-2-Aminoadipate 6-semialdehyde	1.000E-06
C04411	compound	(2R,3S)-3-Isopropylmalate	1.000E-06
C06032	compound	D-erythro-3-Methylmalate	1.000E-06
C16698	compound	N-Acetylmuramic acid 6-phosphate	2.110E-03

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