**RESEARCH ARTICLE**

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**EDITOR’S CHOICE**

*Stenotrophomonas maltophilia* produces an EntC-dependent catecholate siderophore that is distinct from enterobactin

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**Abstract**

*Stenotrophomonas maltophilia*, a Gram-negative, multi-drug-resistant bacterium, is increasingly recognized as a key opportunistic pathogen. Thus, we embarked upon an investigation of *S. maltophilia* iron acquisition. To begin, we determined that the genome of strain K279a is predicted to encode a complete siderophore system, including a biosynthesis pathway, an outer-membrane receptor for ferrisiderophore, and other import and export machinery. Compatible with these data, K279a and other clinical isolates of *S. maltophilia* secreted a siderophore-like activity when grown at 25–37°C in low-iron media, as demonstrated by a chrome azurol S assay, which detects iron chelation, and Arnow and Rioux assays, which detect catecholate structures. Importantly, these supernatants rescued the growth of iron-starved *S. maltophilia*, documenting the presence of a biologically active siderophore. A mutation in one of the predicted biosynthesis genes (entC) abolished production of the siderophore and impaired bacterial growth in low-iron conditions. Inactivation of the putative receptor gene (*fepA*) prevented the utilization of siderophore-containing supernatants for growth in low-iron conditions. Although the biosynthesis and import loci showed some similarity to those of enterobactin, a well-known catecholate made by enteric bacteria, the siderophore of K279a was unable to rescue the growth of an enterobactin-utilizing indicator strain, and conversely iron-starved *S. maltophilia* could not use purified enterobactin. Furthermore, the *S. maltophilia* siderophore displayed patterns of solubility in organic compounds and mobility upon thin-layer chromatography that were distinct from those of enterobactin and its derivative, salmochelin. Together, these data demonstrate that *S. maltophilia* secretes a novel catecholate siderophore.

**INTRODUCTION**

The Gram-negative bacterium *Stenotrophomonas maltophilia* is now recognized as an important opportunistic and nosocomial pathogen [1–3]. Occurring naturally in water, soil and plants, *S. maltophilia* is a member of γ-Proteobacteria and is the most studied of the 15 validated *Stenotrophomonas* species [4–8]. Pneumonia and bacteraemia are the most common manifestations of *S. maltophilia* infection, although *S. maltophilia* is also associated with central nervous system, eye, heart, skin, soft tissue and urinary tract infections [1, 3, 9]. In North America and Europe, the incidence of *S. maltophilia* is on the rise in cystic fibrosis patients [1, 10, 11]. Furthermore, *S. maltophilia* infection in cystic fibrosis patients is an independent risk factor for lung exacerbations [1, 2, 11, 12]. A main reason for this growing problem is the inherent antibiotic resistance of *S. maltophilia* [1, 3, 13]. In spite of the growing significance of *S. maltophilia* infections, our knowledge of *S. maltophilia* pathogenicity is limited. We and others have documented that the instillation of *S. maltophilia* into the lungs of mice results in pneumonia, consisting of bacterial replication, tissue damage and marked inflammation [14–16]. Phenotyping indicates that *S. maltophilia* strains have traits that are linked to virulence in other bacteria [1, 17]. Recently, we found that *S. maltophilia* encodes a type II secretion system that secretes proteases that damage lung epithelia [18–20].

The genomes of clinical and environmental isolates of *S. maltophilia* [21, 22] encode homologues of EntA, EntC and EntF, three proteins that are involved in the biosynthesis of enterobactin (also known as enterochelin), a catecholate siderophore that has an exceptionally high affinity for ferric iron and is made by enteric bacteria (e.g. *E. coli*) and...
Streptomyces sp. [23–25]. Compatible with this finding, many strains of S. maltophilia, including the multi-drug-resistant K279a and other isolates from infected human tissue, secrete a substance (or substances) that is (are) detected by the chrome azurol S (CAS) assay (which detects Fe$^{3+}$ chelating activity independently of chelator structure) and the Arnow assay (which detects the catecholate moiety) [26–29]. S. maltophilia strains are negative in the Csáky assay, indicating that they do not secrete a hydroxymate, which is another type of structure commonly found in siderophores [29]. Based on these various data, it has been inferred that S. maltophilia makes a siderophore and, more specifically, enterobactin [21, 22, 29]; however, bioassays, mutational analysis and direct biochemical comparisons between the S. maltophilia siderophore and enterobactin have been lacking. Here, we describe a larger set of siderophore synthesis and transport genes in S. maltophilia strain K279a, and by mutant analysis confirm a link between two representative genes (i.e. entC and fepA) and siderophore production and utilization. Importantly, multiple bioassays and biochemical analyses reveal that the S. maltophilia siderophore is in fact distinct from enterobactin and appears to have a novel structure.

METHODS

Bacterial strains, media and growth assays

The multi-drug-resistant S. maltophilia K279a (American Type Culture Collection strain BAA-2423) was used as both our wild-type strain and the parental control for newly made mutants [14, 18, 19, 30]. We also examined S. malto- philia strains UPSm1, UPSm2 and UPSm3, which had been obtained from patients beforehand [14, 18]. The S. malto- philia strains and mutants were cultured routinely at 37 °C on Luria–Bertani (LB) agar (Becton Dickinson, Franklin Lakes, NJ, USA) or LB broth. Escherichia coli strain DH5α (Life Technologies, Carlsbad, CA, USA) served as a host strain for the cloning and propagation of recombinant plasmids, and Salmonella typhimurium TA2700 was used as an indicator strain for the detection of enterobactin [31]. The E. coli and S. typhimurium strains were maintained on LB agar or in LB broth at 37 °C, unless otherwise noted. When appropriate, the growth media were supplemented with ampicillin (Research Products International, Mt Prospect, IL, USA) at 100 µg ml$^{-1}$, chloramphenicol at 30 µg ml$^{-1}$, gentamicin at 5 µg ml$^{-1}$, tetracycline at 10 µg ml$^{-1}$ and/or 10 % sucrose. All chemicals were from Sigma-Aldrich (St Louis, MO, USA) unless otherwise noted. In order to assess bacterial growth under iron-restricted conditions, the S. malto- philia strains were monitored for growth in Stainer–Scholte minimal medium that contained casamino acids (SSC) and had been depleted of free iron, as detailed below [29, 32]. After overnight incubation on LB agar at 37 °C, single colonies were inoculated into 3 ml of LB broth (in a 5 ml glass tube) and incubated for 16 h with shaking at 225 r.p.m. (C25KC Incubator Shaker, New Brunswick, Edison, NJ, USA). The resultant cultures were centrifuged at 3619 g for 10 min, and the bacterial pellet was washed twice in 3 ml of PBS and then resuspended to an optical density at 600 nm (OD$_{600}$) of 0.2. Finally, 100 µl of this suspension was inoculated into 50 ml (in a 250 ml acid-washed flask) of SSC medium containing 0, 100, 200, or 400 µM of the ferrous iron chelator 2, 2′-dipyridyl (DIP) (also known as 2, 2′-bipyridyl or 2, 2′-bipyridine) [33–35], and then the culture was incubated at 37 °C with shaking at 225 r.p.m. (C25KC Incubator Shaker). Every 12 h for the next 2 days, bacterial growth was monitored by determining the OD$_{600}$ using a DU 720 spectrophotometer (Beckman Coulter, Indianapolis, IN, USA) [19].

Siderophore assays

In order to examine siderophore production by S. malto- philia, low-free-iron SSC broth cultures (above) were centri- fuged at 3619 g for 30 min at 4 °C, and the resultant supernatants sterilized by passage through 0.22 µm syringe filters (EMD Millipore, Billerica, MA, USA). The cell-free supernatants were tested for siderophore activity (i.e. ferric iron chelation) using the CAS assay as previously described [36–38], after it was confirmed that the DIP contained within the growth medium did not itself react in the CAS assay, even when present at a concentration of 10 000 µM. The levels of CAS reactivity produced by S. malto- philia strains were expressed as 2, 3-dihydroxybenzoic acid (DHBA) equivalents, as calculated from a standard curve generated using a range of concentrations of purified DHBA. To detect the catecholate structure, the Arnow and Rioux assays were performed [37, 39–41]. As before, DHBA and enterobactin served as standards for these assays. To judge the growth-promoting ability of the S. malto- philia siderophore, we developed an agar plate-based bioassay. To that end, 50 µl of supernatant obtained from SSC cultures containing 400 µM DIP was spotted unto a 6 mm paper disc (Becton Dickinson Co., Sparks, MD, USA) placed at the centre of a low-iron agar plate [i.e. SSC medium with 200 µM DIP and 0.5 mg l$^{-1}$ FeSO$_4$ added and the % agar (US Biological Life Sciences) reduced from 1.5 to 0.5 %], onto which K279a bacteria had been spread, and the stimula- tion of bacterial growth was assessed after 3 d of incuba- tion at 37 °C. As a positive control, we added 50 µl of 0.5M FeCl$_3$ to the paper discs. The ability of enterobactin and salmochelin (EMC Microcollections GmbH, Tübingen, Germany) to stimulate K279a was tested by suspending the purified siderophores in low-free-iron SSC media and spotting various amounts onto the paper discs. In a similar way, we tested the growth-stimulating ability of enterobactin and salmochelin byproducts, i.e. monomers and linear dimers and trimers, using ENB mix and Salmochelin mixture obtained from EMC Microcollections. To assess the ability of the S. malto- philia siderophore to stimulate the growth of an enterobactin indicator strain, we spotted the same culture supernatants onto discs placed in the centre of an M9 minimal medium (0.5 %) agar plate [31], onto which S. typhimurium strain TA2700 had been spread [31]. To prepare the K279a and TA2700 strains for inclusion on the bio- assay plates, bacteria were first cultured on LB agar overnight at 37 °C, and then single colonies were inoculated
into LB broth and incubated overnight with shaking at 225 r.p.m. After the log-phase bacteria were resuspended in PBS to an OD<sub>600</sub> of 0.5, 100 µl was spread-plated onto the low-iron bioassay media using sterile cotton swabs.

**Organic extraction and chromatography of siderophores**

In order to judge the solubility of the <i>S. maltophilia</i> siderophore in organic compounds, K279a was grown in SSC medium containing 400 µM DIP as described above, and 10 ml of cell-free culture supernatant wasacidified by the addition of 50 µl of 2 N HCl. Two 4 ml aliquots of the acidified supernatant were then separately mixed with 4 ml of ethyl acetate, dichloromethane, or butanol by shaking at 225 r.p.m. (C25KC Incubator Shaker) for 5 min at 25°C. Following centrifugation at 3619 g for 5 min, the aqueous phases from the two samples were combined. The extraction efficiency was finally determined by comparing the CAS activity in the acidified supernatant with the activity present in the extracted aqueous phase. In order to compare the solubility of the K279a siderophore to that of known siderophores, 50 µl of purified enterobactin or salmochelin were suspended in 1 ml of the SSC medium, acidified and then extracted in the manner just described. In preparation for thin-layer chromatography (TLC) analysis of the <i>S. maltophilia</i> siderophore and enterobactin, 1 ml of the organic phases obtained by ethyl acetate extraction as above was dried in a 60 Hz Savant SpeedVac DNA 100 concentrator (Thermo Fisher Scientific, Waltham, MA, USA) at 65°C for 4 h. The resulting pellets were dissolved in 20 µl of methanol, and then 10 µl of the solution was spotted onto TLC aluminum sheets with silica gel 60 WF254 (EM Science, Gibbstown, NJ, USA) and developed for 45 min with butanol:glacial acetic acid:water (5:2:2 v/v/v), as previously described [42]. Finally, the plates were sprayed with 5% FeCl<sub>3</sub> in order to visualize the migration of the iron-binding species. TLC analysis of salmochelin was performed in the same way, except that this siderophore was dissolved directly in methanol prior to TLC.

**DNA and protein sequence analysis**

<i>S. maltophilia</i> DNA was isolated as before [18]. The primers used for sequencing and PCR were obtained from Integrated DNA Technologies (Corvalle, IA, USA) and are listed in Table S1 (available in the online Supplementary Material). DNA and protein sequences were analysed using Lasergene (DNASTAR, Madison, WI, USA). BLASTN and BLASTP homology searches were performed using GenBank at the NCBI. For EntC and FepA alignments, we obtained FASTA formats from NCBI and then generated alignments using both T-Coffee (http://tcoffee.crg.cat/apps/tcoffee/do: regular) and Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Putative Fur boxes within the promoter regions of the <i>S. maltophilia</i> ent and fep genes were identified using the MART tool within the MEME suite (http://meme-suite.org/tools/mast) [43, 44].

**Mutation construction and genetic complementation**

The entC and fepA mutants of strain K279a were constructed using a recombineering approach through <i>E. coli</i> and Flp excision as described previously [45]. The entC mutants NUS8 and NUS9 had a deletion of the entire entC coding region (SMLT_RS13420). The entC gene, along with 600 bp of flanking DNA on each side, was PCR-amplified from K279a DNA using primers MN1 and MN2 and Platinum Pfx polymerase (Life Technologies, Carlsbad, CA, USA), and ligated into pGEM-T Easy (Promega, Madison, WI, USA), creating the plasmid pGentC. An Flp recombination target (FRT)-flanked chloramphenicol cassette was PCR-amplified from pKD3 [45] using primers MN3 and MN4 and High Fidelity Taq (Life Technologies). Fifty ng of pGentC DNA dissolved in 3 µl of nuclease-free water (Promega, Madison, WI, USA) and 150 ng of the FRT-flanked Cm cassette dissolved in 7 µl of nuclease-free water were added to 50 µl of induced electro-competent DY330, an <i>E. coli</i> strain with a red recombinase genes [45]. Following electroporation of the mixture at 2500 V using the GenePulserXcell (BioRad, Hercules, CA, USA), the cells was transferred into 1 ml of SOC medium [46] and incubated at 30°C with shaking. After 6 h, the entire sample was plated onto chloramphenicol-containing LB agar, and colonies were recovered after 2 days of incubation at 30°C. After the plasmid within the colonies was verified by PCR using primers MN1 and MN2, it was transferred into strain DH5α. The construct was then digested with BamHI and HindIII and ligated into pEX18Tc [19] digested with the same enzymes, yielding pEXAentC::frt-Cm-frt. The newly made plasmid was moved into <i>E. coli</i> S17-1 and mobilized from there into <i>S. maltophilia</i> K279a via conjugation, as previously described [19]. To select for <i>S. maltophilia</i> carrying the mutagenizing construct that provides resistance to tetacycline from the vector backbone and resistance to chloramphenicol from the mutated gene, transconjugants were plated on LB agar supplemented with tetacycline, chloramphenicol (10 µg ml<sup>-1</sup>) and norfloxacin. Since K279a is resistant to norfloxacin [18], the inclusion of this antibiotic selects against outgrowth of the S17-1 strain. Resistant colonies were plated onto LB agar supplemented with 10% sucrose and chloramphenicol to select for cells in which recombination and loss of the pEX18Tc vector has occurred. The fepA mutant NUS10 had a deletion of the entire fepA coding region (SMLT_RS06850) and was obtained using a similar approach. Primers MN5 and MN6 were used to obtain the starting material, i.e. fepA along with 600 bp flanking DNA on each side, for cloning into pGEM-T Easy. Primers MN7 and MN8 were used to amplify the FRT-flanked chloramphenicol cassette from pKD3. To achieve Flp-mediated excision of the chloramphenicol gene in the entC and fepA mutants, pBSFlp [45] was electroporated into the mutants and transformants were selected on LB agar containing gentamicin and IPTG (1 mM). Following overnight incubation at 37°C, the plates were incubated at room temperature for another 24 h, at which time the small colonies that appeared were patched onto LB agar containing...
chloramphenicol, gentamicin, or no selection. Colonies that were either gentamicin- or chloramphenicol-sensitive were streaked onto sucrose-containing LB agar at 37°C overnight, and the desired gene deletions were confirmed by PCR using the primer pairs MN1 and MN2 for entC or MN5 and MN6 for fepA.

For trans-complementation of entC mutant NUS8, a 1.5 kb PCR fragment containing the entC-coding region plus its promoter was PCR-amplified from K279a DNA using primers MN9 and MN10. To complement fepA mutant NUS10, a 2.3 kb PCR fragment encompassing the fepA-coding region and promoter was amplified from K279a DNA using primers MN11 and MN12. The entC and fepA fragments were each digested with KpnI and XbaI and cloned into pBBR1MCS [19, 47] digested with the same enzymes, yielding pBentC and pBfepA. Plasmids pBentC and pBfepA were electroporated [18] into the mutant and fepA mutants, respectively, and transformants were selected on LB agar containing chloramphenicol. Clones were confirmed as carrying pBentC or pBfepA by PCR using primers MN9 and MN10, or MN11 and MN12, respectively.

**RESULTS**

*S. maltophilia* appears to encode a complete system for siderophore production and utilization

An examination of the genome of the *S. maltophilia* clinical isolate K279a [48] revealed the presence of eight loci, which, when combined, are predicted to encode a system for siderophore production and utilization. The first locus had six open-reading-frames (ORFs) predicted to encode proteins that share similarity with the enterobactin biosynthesis enzymes; i.e. RS13395 is related to EntA, RS13400 EntF, RS13405 EntD, RS13410 EntB, RS13415 EntE and RS13420 EntC (Table 1). In enterobactin synthesis, (1) EntC converts chorismic acid to isochorismate, (2) EntB converts isochorismate into 2, 3-dihydroxy-2, 3-dihydroxybenzoate, (3) EntA converts 2, 3-dihydroxy-2, 3-dihydroxybenzoate into DHBA, and (4) EntE, EntF and EntD join DHBA and serine to make enteroactin [23, 49]. Although the level of similarity to EntD is less than that observed with the other Ent proteins, EntD and its related proteins are Sfp-type phosphopentadentyl transferases that can be involved in various biosynthetic pathways in addition to siderophore synthesis [50–52]. Thus, strain K279a appeared to have all of the genes needed to make enterobactin or an enterobactin-related molecule. *S. maltophilia* also seemed to have the machinery needed to secrete a siderophore. RS13425, directly upstream of entC, is predicted to encode an inner-membrane transporter belonging to the major facilitator superfamily [42, 53], and ORF RS18720 encodes TolC [54, 55], which, in other bacteria, mediates siderophore export across the outer membrane [56–58] (Table 1). Finally, the K279a genome indicated that *S. maltophilia* has the factors needed for siderophore uptake (Table 1), i.e. (i) RS06850 encodes an outer-membrane protein that has similarity to the enterobactin receptor FepA of *E. coli* and related proteins, such as PfeA and PirA of *Pseudomonas aeruginosa* [23, 59–61]; (ii) RS21345, RS18555 and RS00050 encode TonB, ExbB and ExbD, proteins that form a periplasmic-spanning complex that provides the energy for the opening of the outer-membrane receptor channel, thereby allowing ferri-siderophore to enter into the periplasm [23, 62–64]; and (iii) ORFs RS11320, RS11325 and RS11330 encode

<table>
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<tr>
<th>Locus</th>
<th>ORF*</th>
<th>Annotated gene name</th>
<th>Relatedness to <em>E. coli</em> protein (percentage similarity, percentage identity, E value)</th>
<th>Putative function</th>
<th>Putative location</th>
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*NCBI GenBank accession number: AM743169.1.
†IM, inner membrane; OM, outer membrane.
proteins with similarity to FepC, FepD and FepG, which mediate movement of ferri-enterobactin from the periplasm to the cytoplasm [23]. BLAST analysis did not reveal a *S. maltophilia* protein with similarity to EntS, an outer-membrane exporter of enterobactin, nor did it identify a homologue of either FepB, a periplasmic protein that associates with ferri-enterobactin during transport, or Fes, the cytoplasmic esterase that cleaves ferri-enterobactin and thereby promotes the ultimate release of iron [23, 65–67]. However, we did find that ORF RS02330 has similarity with YgiH and ViuB (Table 1), which are cytoplasmic reductases that aid in the release of iron from other siderophores [68]. All of the other 12 sequenced strains of *S. maltophilia* examined harboured the set of the siderophore-related genes first found in K279a (Table S2), signalling that siderophore metabolism is conserved in the species. The presence of many *ent*- and *fep*-related genes does suggest that the *S. maltophilia* siderophore is enterobactin; however, the levels of sequence similarity to the *E. coli* prototypes and the lack of a complete set of enterobactin-associated genes could mean that *S. maltophilia* is making a variant of enterobactin or a siderophore that is distinct from enterobactin. K279a lacks an orthologue of IroB, the salmochelin glucosyltransferase, or any of the other proteins needed for salmochelin export or utilization [69]. Thus, we posited that *S. maltophilia* might be producing a novel siderophore.

The presence of the *ent*- and *fep*-related genes in other sequenced species of *Stenotrophomonas* [70] is reported in Table S3. *S. pavanii* encoded a full set of proteins that had >90% similarity to the Ent- and Fep-related proteins of

![Fig. 1](https://www.microbiologyresearch.org/article/163/1590-1603/fig1.png)

**Fig. 1.** Kinetics and iron-dependence of siderophore production by *S. maltophilia*. (a) Strain K279a bacteria were inoculated into SSC medium containing, as indicated, 0, 100, 200, or 400 μM DIP, and then incubated at 37 °C for 2 days. Bacterial growth was recorded spectrophotometrically (left y axis), and the CAS reactivity of culture supernatants, which is measured as net DHBA equivalents, was monitored (right y axis). The values presented are the means and standard deviations from triplicate cultures, although small error bars are not always visible over the symbols. The ODS of the cultures containing 400 μM DIP were significantly smaller than the others at all of the time points post-inoculation, whereas the growth of the cultures containing 200 μM DIP was smaller than that of the 0 and 100 μM DIP cultures at 24, 36 and 48 h (Student’s t-test; P<0.05). The data are representative of three independent experiments. (b) A comparison of the levels of CAS reactivity associated with the different culture conditions in (a) at the indicated time points. Asterisks indicate significant differences between samples (Student’s t-test; *, P<0.05; **, P<0.005).
S. maltophilia, while for S. rhizophila, the levels of similarity were 60–85%. However, for the other eight species examined, the levels of similarity to the K279a prototypes were significantly reduced and more variable. These data suggested that some of the other Stenotrophomonas species produce the same siderophore as S. maltophilia.

**Kinetics of siderophore expression by K279a and other S. maltophilia strains**

To begin our experimentation, we sought to use the CAS assay [36, 37] to measure, for the first time, the levels of secreted siderophore activity over the full course of strain K279a’s growth in broth culture. To that end, K279a was cultured in media in which the levels of free iron available to the bacterium were reduced by the inclusion of DIP, which is a ferrous iron chelator that does not react in the CAS assay, as has been done before when examining a variety of bacteria [29, 71–73]. Upon the incubation of strain K279a in the low-free-iron SSC media at 37 °C with shaking, CAS reactivity appeared in the log phase at 12 h post-inoculation and then steadily increased over the next 24 h, along with bacterial numbers (Fig. 1a). The levels of reactivity rose in parallel with decreasing levels of free iron (i.e. increasing concentrations of DIP) at every time point examined (Fig. 1b). Compatible with there being iron-mediated repression, we observed a putative Fur-binding site (‘Fur box’) upstream of the operon encoding the MFS transporter and Ent-related biosynthetic enzymes that is identical in 11 out of the 19 bases to the E. coli Fur box consensus and, in agreement with a recent in silico analysis of Fur boxes in the S. maltophilia K279a genome [61] (Fig. S1a). Unsurprisingly, bacterial growth slowed as the levels of free iron decreased (Fig. 1a). The CAS reactivity of the cell-free culture supernatants was stable upon 7 days of further incubation at 37 °C, implying that S. maltophilia had not secreted an enzyme that degrades the siderophore activity.

Since S. maltophilia is mainly an environmental organism, we examined the siderophore levels in cultures that had been grown at lower temperatures. The growth of K279a was slower at 30 and 25 °C compared to at 37 °C, especially in media with lower quantities of free iron (Fig. 2, left panels). Siderophore was still detected at the lower temperatures (Fig. 2, right panels), although the emergence of CAS reactivity was delayed and the levels of activity were lower,
undoubtedly due to the slowed bacterial growth at 30 and 25 °C. These data suggest that S. maltophilia produces its siderophore(s) when growing in both the human host and in environmental niches.

CAS-positive culture supernatants were obtained when three other clinical isolates of S. maltophilia were examined (Fig. S2a). All of the supernatants were also positive in the Arnow assay [37, 39] (Fig. S2b), indicating the presence of a catecholate siderophore. Strain UPSm2 expressed a level of siderophore activity that was greater than that of the other three strains examined. In sum, our results confirmed that S. maltophilia secretes an iron-responsive siderophore activity, which, at the least, includes a catecholate. Since enterobactin and its variants are catecholates that are reactive in the CAS and Arnow assays [23, 37], these data were compatible with the in silico analysis.

**Mutation of entC abolishes siderophore production by strain K279a**

As a next step towards defining the S. maltophilia siderophore, we mutated ORF RS13420 (Table 1), which is predicted to encode a homologue of EntC, the first enzyme in the enterobactin pathway in other bacteria [74, 75]. An alignment of the K279a protein with E. coli EntC appears in Fig. S3a. The inactivation of entC consisted of a deletion of the entire ORF and was achieved using allelic exchange, as we have done for other S. maltophilia mutants [18, 19]. When grown at 37 °C in SSC media containing 100 or 200 µM DIP, the entC mutant NUS8 exhibited no CAS reactivity (Fig. 3a, left panels). Reintroduction of entC into NUS8 restored siderophore activity to wild-type levels (Fig. 3a, left panels), confirming the necessity of entC for siderophore production. The entC mutant, but not its complement, also lost reactivity in the Arnow assay and the Rioux assay, which also detects catecholate structures (Fig. 3b). A second, independently derived entC mutant (NUS9) also lacked siderophore activity (not shown). Because the entC mutants had a loss in CAS reactivity that was comparable in magnitude to the loss in Arnow and Rioux reactivity, we inferred that the three reactivities are a manifestation of a single molecular species. The entC mutant, but not its complement, showed the same loss in siderophore activity at 30 and 25 °C (Fig. S4), indicating that this molecular species is made at all of the examined temperatures. Finally, since the entC mutant had no residual siderophore activity at any temperature or level of free-iron
depletion, it appears that strain K279a only expresses one siderophore, at least in the growth medium used here. Overall, these data provide the first proof of a link between the entAFDBEC operon and the production of the S. maltophilia siderophore.

Wild-type K279a supernatants, but not entC mutant supernatants, rescue the growth of iron-starved S. maltophilia

To assess the biological significance of the S. maltophilia siderophore activity, we examined the ability of wild-type supernatants to rescue the growth of iron-starved bacteria by developing a bioassay akin to that which we and others have used to study other siderophores [25, 37, 38, 76]. Thus, an aliquot of the CAS+, Arnow+, Rioux+supernatant obtained from cultures of strain K279a was placed onto a disc in the centre of a low-free-iron SSC agar plate, onto which we had spread K279a bacteria. Unlike a media-alone control, inclusion of the wild-type sample stimulated growth around the well (Fig. 3c), as also occurred when FeCl$_3$ was put onto a disc. When entC mutant NUS8 supernatants were tested, there was no growth stimulation (Fig. 3c). Yet, supernatants obtained from the complemented entC mutant behaved like the wild-type supernatants did (Fig. 3c). These data documented that the S. maltophilia siderophore has growth-promoting activity. Supporting this conclusion, the entC mutant, but not its complement, exhibited impaired growth when it was inoculated into a medium that had an especially low level of free iron due to the inclusion of 400 µM DIP (Fig. 3a).

Mutation of fepA abolishes siderophore usage by strain K279a

As noted in Table 1, the genome of strain K279a carries a gene, RS06850, which is predicted to encode an outer-membrane protein that has similarity to the TonB-dependent enterobactin receptor FepA. An alignment of the S. maltophilia protein with E. coli FepA appears in Fig. S3b. Upstream of ORF RS06850 (previously annotated as fepA) is a putative Fur box (Fig. S1b), which is compatible with the gene being subject to Fur- and iron-mediated repression [61]. When its fepA gene was mutated, K279a lost the ability to utilize CAS+, Arnow+ and Rioux+supernatants for growth stimulation on low-iron media (Fig. 4a, top panel). Because the fepA mutant was still able to utilize added FeCl$_3$, it does not have a generalized defect in iron uptake. A complemented derivative behaved like parental wild-type did in the siderophore bioassay (Fig. 4a, bottom panel). Taken together, these
results demonstrated, for the first time, that the product of ORF RS06850 is required for siderophore utilization and, based on its predicted outer-membrane location and similarity to *E. coli* FepA, it is most likely the receptor for the *S. maltophilia* siderophore. Strain K279a encodes additional TonB-dependent receptors that are iron-regulated (e.g. ORF RS19685) [61]; however, we do not believe that they are especially pertinent here, because the loss of *fepA* (ORF RS06850) abolished siderophore utilization in the bioassay. Following *fepA* mutant incubation in deferrated SSC media, its supernatant exhibited a slight increase in CAS reactivity relative to wild-type K279a and its complement (Fig. 4b), which is indicative of a bacterial population that is experiencing iron starvation due to the loss of siderophore receptor. Overall, the phenotype of the *fepA* mutant, coupled with the results obtained by analysing the *entC* mutant, further suggested that *S. maltophilia* elaborates either enterobactin or an enterobactin-related molecule.

**The *S. maltophilia* siderophore is distinct from enterobactin and the enterobactin-derivative salmochelin**

Having shown that *S. maltophilia* expresses and utilizes a biologically active siderophore, we sought to determine whether the molecule is in fact identical to the catecholate enterobactin. First, we tested the ability of K279a supernatants to rescue the growth of a *Salmonella typhimurium* enterobactin-indicator strain (TA2700) on a low-iron medium [31, 77–81]. Although the growth of TA2700 was, as expected, readily rescued by the addition of purified enterobactin, it was not restored after exposure to the K279a supernatants, even though those supernatants had as much CAS activity as the enterobactin control (Fig. 5a). The inability of the supernatants to restore growth was not an artifact of any potential inhibitory substance in the sample, since the purified enterobactin that did stimulate growth had been suspended in culture supernatants. These

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**Fig. 5.** Comparison of the *S. maltophilia* siderophore and enterobactin regarding their ability to stimulate bacterial growth and their extraction by organic solvents and migration on TLC. (a) *S. typhimurium* strain TA2700 was spread across the surface of a low-iron M9 minimal medium plate and a paper disc placed in the centre of the plate was infused with low-iron SSC medium, enterobactin suspended in the SSC medium and supernatant obtained from WT K279a grown in SSC medium containing 400 µM DIP. After 3 days of incubation at 37°C, bacterial growth around the disc was recorded. (b) Supernatants obtained from WT K279a grown in SSC medium containing 400 µM DIP or purified enterobactin suspended in the SSC medium were subjected to extraction by ethyl acetate, dichloromethane, or butanol, after which the levels of CAS reactivity in the aqueous phases before and after extraction were determined. The values presented are the means and standard deviations from triplicate samples. Asterisks indicate a significant difference between the before- and after-extraction readings (Student’s t-test; ***, P<0.005). (c) Following extraction with ethyl acetate, enterobactin and supernatants obtained from either WT K279a, *entC* mutant NUS8, or complemented mutant NUS8 (pB*entC*) grown in SSC medium containing 400 µM DIP were subjected to TLC. Upon spraying FeCl₃ onto the developed plate, the iron-binding species in each sample was identified (circles). The left arrow indicates the direction of migration, with the line towards the bottom of the plate denoting the starting point for the migration. The arrow to the right shows the direction of increasing polarity. (d) WT bacteria were spread across the surface of a low-iron SSC plate and a disc on the plate was infused with low-iron SSC medium, supernatant obtained from WT grown in SSC medium containing 400 µM DIP, or the indicated amounts of purified enterobactin suspended in the SSC medium. After incubation at 37°C, bacterial growth was recorded. For (a–d), the data presented are representative of three independent experiments.
data provided a strong indication that *S. maltophilia* is not producing enterobactin. Supporting this conclusion, purified enterobactin and the K279a sample behaved differently when they were subjected to extraction with organic solvents; i.e. whereas enterobactin was easily extracted into ethyl acetate, butanol and dichloromethane, the *S. maltophilia* activity was extracted into ethyl acetate but not butanol and dichloromethane (Fig. 5b). Furthermore, when ethyl acetate-extracted samples were subjected to TLC [42], the K279a supernatant contained an iron-binding species that had a mobility that was distinct from enterobactin (Fig. 5c). That this species was absent from supernatants of the *entC* mutant (but not its complement) confirmed that it was the *S. maltophilia* siderophore (Fig. 5c). That the K279a siderophore did not migrate as far as enterobactin did indicated that the molecule is more polar than enterobactin. As yet additional evidence for the dissimilarity between enterobactin and the *S. maltophilia* siderophore, purified enterobactin (i.e. cyclic trimer), given in a range of amounts, was unable to rescue the growth of strain K279a on a low-iron medium (Fig. 5d). Furthermore, a mixture of enterobactin, its monomer 2, 3-dihydroxybenzoylserine (DHBS), the linearized dimer (DHBS)2 and linearized trimer (DHBS)3 [82], although capable of rescuing the growth of *S. typhimurium* enterobactin-indicator strain, as expected, did not stimulate the growth of *S. maltophilia* WT K279a or its *entC* mutant and *fepA* mutant derivatives (Fig. S5). In sum, multiple lines of proof document that the *S. maltophilia* siderophore is actually distinct from enterobactin.

Although, as noted above, strain K279a does not encode a homologue of IroB, the enzyme that converts enterobactin into salmochelin, we nonetheless compared the *S. maltophilia* siderophore to purified salmochelin in both our bioassays and chemical extraction and separation procedures. Even though salmochelin was able to rescue the growth of iron-starved *S. typhimurium*, as expected (not shown), it did not stimulate the growth of strain K279a on a low-iron medium (Fig. 6a). Furthermore, a mixture containing

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**Fig. 6.** Comparison of the *S. maltophilia* siderophore with salmochelin. (a) WT K279a bacteria were spread across the surface of a low-iron SSC plate and a paper disc placed in the centre of the plate was infused with deferrated SSC medium, supernatant obtained from K279a grown in SSC medium containing 400 µM DIP, or the indicated amount of purified salmochelin suspended in the SSC medium. After 3 days of incubation at 37°C, bacterial growth was recorded. (b) Supernatants obtained from WT K279a grown in SSC medium containing 400 µM DIP or purified salmochelin suspended in the SSC medium were subjected to extraction by ethyl acetate, dichloromethane, or butanol, and then the levels of CAS reactivity in the aqueous phases before and after extraction were determined. The values presented are the means and standard deviations from triplicate samples. Asterisk indicates a significant difference between the before- and after-extraction readings (Student’s t-test; **, *P*<0.005). (c) Salmochelin and enterobactin and supernatants that were obtained from WT K279a grown in deferrated SSC medium were subjected to TLC. The iron-binding species in each sample are marked by circles. The left arrow indicates the direction of migration, and the arrow to the right shows the direction of increasing polarity. The data presented in (a–c) are representative of three independent experiments.
salmochelin, its linearized hydrolysis product, and a dimer of DHBA (glucosyl)-seryl-DHBA-serine [83], although able to stimulate the growth of the Salmonella indicator strain, did not stimulate S. maltophilia growth (Fig. S5). Finally, salmochelin did not extract into organic solvents in the way that the K279a siderophore did (Fig. S6b), nor did it migrate as the S. maltophilia siderophore did during TLC (Fig. 6c). Thus, we conclude that the S. maltophilia is also different from salmochelin, the best characterized derivative of enterobactin.

DISCUSSION

This study is the first combined bioinformatic, genetic and biochemical characterization of a S. maltophilia siderophore. Unsurprisingly, the siderophore was produced during bacterial growth in low-iron media, likely linked to derepression via Fur at the ent- and fep- containing loci (Fig. S1). Furthermore, we demonstrated that the CAS+, Arnow +, Rioux+ substance that is secreted by strain K279a is able to promote bacterial growth in low-iron conditions, thereby documenting its biological activity and completing its formal definition as a bona fide siderophore. Our data prove that S. maltophilia does not produce enterobactin (or salmochelin), as previously indicated in the literature, but rather secretes a catecholate that appears to be novel in structure. On the one hand, given the presence of genes related to entA, entB, entC, entD, entE and entF in the K279a genome, it is plausible that the Stenotrophomonas siderophore, though unique, is derived from enterobactin, such that there are one or a few modifications to the enterobactin backbone. In this case, we would predict that there is an additional (novel) biosynthesis gene that is unlinked to the K279a entC-containing locus. On the other hand, it is conceivable that the S. maltophilia siderophore bears little resemblance to enterobactin and therefore S. maltophilia EntC and the other Ent proteins are catalyzing unique reactions. Although the precise identity of the siderophore will require extensive MS and NMR analyses, our current data do provide clues regarding its structure. Based upon TLC and organic extractions, the siderophore is more polar than enterobactin, but less polar than salmochelin. Also, given its stronger reaction in the Rioux assay relative to the Arnow assay, the siderophore would appear to have a modification at position-3 and/or position-4 in the catecholate structure [41, 84]. In addition to likely having a novel structure, the S. maltophilia siderophore may be unique in terms of its iron assimilation within the bacterial cell, given that strain K279a lacks proteins similar to FepB and the Fes esterase.

Although many advances have been made in understanding S. maltophilia’s antibiotic resistance [3, 85, 86], few studies have defined its virulence factors [18, 19, 87–89]. Thus, identifying aspects of iron acquisition is significant when one considers the present state of the Stenotrophomonas field. The role of siderophores in disease has been shown for many bacteria, including pathogens of the lung and the blood, the most common sites for S. maltophilia infection [66, 76, 90–92]. Among the siderophores that promote infection are enterobactin, DHBA and salmochelin [23, 66, 69, 90, 93, 94]. It is also important to note that siderophore secreted by one microbe can be ‘stolen’ by others; e.g. enterobactin or its variants are used (but not made) by a variety of bacteria [76, 95, 96]. Thus, a S. maltophilia siderophore might be used by other microbes that co-exist with S. maltophilia, including P. aeruginosa, the most notorious co-inhabitant of the cystic fibrosis lung [2, 59, 97–100]. Besides promoting bacterial iron assimilation and growth, siderophores influence disease by modulating cytokine/chemokine release from host cells, impeding neutrophil myeloperoxidase and triggering the death of host cells [71, 101, 102]. Based upon these many studies, the S. maltophilia siderophore is likely important in pathogenesis; however, it will be necessary to examine siderophore mutants in an animal model of S. maltophilia disease to confirm this hypothesis.

The pursuit of this newly defined siderophore will not only advance our understanding of S. maltophilia biology and pathogenesis, but it may reveal potential new targets for anti-microbial therapies. Indeed, given its role in disease, siderophore synthesis is often cited as a target for potential new anti-microbials [97, 103–107]. Additionally, discovering a new type of siderophore can have broader significance, since ‘new’ siderophores have potential clinical (as well as industrial) applications as agents to treat iron overload or as carriers for antibiotics [103, 105, 107, 108].

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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