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Siderophore-Mediated Iron Sequestering by *Shewanella putrefaciens*

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The iron-sequestering abilities of 51 strains of *Shewanella putrefaciens* isolated from different sources (fish, water, and warm-blooded animals) were assessed. Thirty strains (60%) produced siderophores in heat-sterilized fish juice as determined by the chrome-azurol-S assay. All cultures were negative for the catechol-type siderophore, whereas 24 of the 30 siderophore-producing strains tested positive in the Csáky test, indicating the production of siderophores of the hydroxamate type. Siderophore-producing *S. putrefaciens* could to some degree cross-feed on the siderophores of other *S. putrefaciens* strains and on compounds produced by an *Aeromonas salmonicida* strain under iron-limited conditions. The siderophores of *S. putrefaciens* were not sufficiently strong to inhibit growth of other bacteria under iron-restricted conditions. However, siderophore-producing *Pseudomonas* bacteria were always inhibitory to *S. putrefaciens* under iron-limited conditions. Growth of siderophore-producing strains under iron-limited conditions induced the formation of one major new outer membrane protein of approximately 72 kDa. Two outer membrane proteins of approximately 53 and 23 kDa were not seen when iron was restricted.

In most aerobic ecological niches, ferric iron (as a free molecule) occurs only in very low concentrations due to the low solubility constant of $Fe(OH)_3$ ($K_{sol} = 10^{-38}$). Most organisms have developed efficient uptake and/or transport systems, and in warm-blooded animals iron is secured in transport molecules like transferrin, allowing the host to control its supply of iron. The majority of microorganisms rely on production of iron-chelating ferric-iron-specific siderophores for their iron supply (34). Siderophores have been shown to play an important role in microbial interactions in the rhizosphere (28) and are also important virulence factors for many microorganisms, allowing the pathogen to establish itself in the host (10, 20, 42).

The siderophores of *Pseudomonas* spp. have been extensively studied, and their siderophores are in some cases important in disease control in the rhizosphere (7). Aquatic *Pseudomonas* spp. have been shown to have a strong inhibitory activity against several microorganisms (26), but no mechanism was suggested for this antibacterial effect. Recently, it was reported that aquatic pseudomonads (5) and *Pseudomonas* spp. isolated from fresh and spoiled fish were strong producers of siderophores and were capable of inhibiting growth of several gram-positive and gram-negative bacteria under iron-limited conditions (17). Particularly, the inhibitory activity against another fish spoilage bacterium, *Shewanella putrefaciens*, was explained by iron competition (17).

S. putrefaciens is a gram-negative bacterium occurring in many ecological niches. It is important for the turnover of inorganic material in sediments (33) and plays an important role in the spoilage of fish (18, 27), poultry (8), and high-pH meat (16). *S. putrefaciens* is a strict aerobic bacterium and has attracted wide interest for its ability to use a large variety of compounds in anaerobic respiration (29). Particularly, its ability to reduce trimethylamine (TMA) oxide to TMA attests to its importance in fish spoilage, and the concentration of TMA has been suggested as a spoilage index for a number of fish species (37). However, in some tropical fish species TMA cannot be used as a spoilage index, as the microflora on iced fish is dominated by *Pseudomonas* spp., which do not reduce TMA oxide. In quality control of fish raw material it is important to know if a TMA-producing microflora or a non-TMA-producing microflora develops. The competition between these groups (i.e., *S. putrefaciens* and *Pseudomonas* spp.) may be related to iron competition (17), and while the high-affinity iron uptake system in *Pseudomonas* spp. has been extensively studied, no reports on the iron-sequestering capabilities of *S. putrefaciens* have been published. The purpose of the present study was to investigate aerobic growth of *S. putrefaciens* under iron-limited conditions.

MATERIALS AND METHODS

Bacterial strains. Fifty-one strains of *S. putrefaciens* isolated from different sources were used (Table 1). Six strains were from culture collections, and of these one (ATCC 8071) was the type strain. Two strains were received from Biolog Inc., and 32 strains were kindly donated by different laboratories (Table 1). Eleven strains were isolated at our laboratory from different fish products. All strains were tentatively identified as *S. putrefaciens*, being motile gram-negative rods, catalase- and oxidase-positive, and positive for reduction of TMA oxide, production of H_2S, and hydrolysis of DNA (47). Inhibitory activity was tested against *Escherichia coli* (ATCC 25922), *Listeria monocytogenes* Scott A isolated from an outbreak of listeriosis (from Campden Food and Drink Association, Chipping Campden, United Kingdom), *Staphylococcus aureus* received from the Royal Veterinary and Agricultural University, Copenhagen, Denmark, *Aeromonas sobria* (a clinical isolate, M2, from S. Knøchel [24]), *Pseudomonas fluorescens* isolated from spoiled Nile perch (*Lates niloticus*) (19), *S. putrefaciens* (ATCC 8071), *Vibrio anguillarum* (from J. L. Larsen, Royal Veterinary and Agricultural University), and *Aeromonas salmonicida* (from the Department of Microbiology and Plant Physiology, University of Bergen, Bergen, Norway). Cross-feeding was tested with *S. putrefaciens* strains, the *P. fluorescens*

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TABLE 1. Origins and siderophore production of *S. putrefaciens* strains

Origin(s)	No. of strains[a]		Source and/or reference[b]
	Producing (30)	Nonproducing (21)	
Lakes, rivers, seawater	1[c]		ACAM 341
	6[d]	1	M. Gennari
Newly caught Mediterranean fish	3	7	M. Gennari
Various fish samples	1	1	B. R. Jørgensen
	1		V. F. Jeppesen
	2	2	47
		1	M. Gennari
	1		S. Suzuki[e]
Spoiled vacuum-packed cod	2[c]	3	B. R. Jørgensen
Spoiled pickled halibut		3	B. R. Jørgensen
Various foods[f]	1		ATCC 8071
		1	NCIMB 10473
		1	ACAM 342
	1		P. Bouvet (41)
	2[c]	1	M. Gennari
Feces	1		NCIMB 11157
Human wounds	2		P. Bouvet (41)
Flamingos	3		P. Bouvet (41)
Oil brine	1		NCTC 10735
Unknown	2		Biolog

[a] Totals are given in parentheses.
[b] ACAM, Australian Collection of Antarctic Microorganisms; ATCC, American Type Culture Collection; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom; NCTC, National Collection of Type Cultures, London. Sources: M. Gennari, Milan, Italy; B. R. Jørgensen and V. F. Jeppesen, Technological Laboratory; S. Suzuki, Hokkaido, Japan; P. Bouvet, Paris, France.
[c] One strain was only weakly positive in the CAS assay.
[d] Three strains were only weakly positive in the CAS assay ($-0.4 < OD_{630} < -0.9$).
[e] The strain was isolated by M. Okuzumi.
[f] Chicken, veal, cheese, and butter.

strain, the *A. salmonicida* strain, and a *P. aeruginosa* strain from Mediterranean fish isolated by M. Gennari, Milan, Italy.

Media. Bacteria were cultured on iron agar (IA) (Oxoid CM 867) and grown in nutrient broth (Difco veal infusion broth). Siderophore production was assessed in heat-sterilized fish juice (13) in which phosphate was omitted, in modified iron broth (MIB) (17), in CM9 (6) (0.2% Casamino Acid-supplemented M9 [43]), and in the basal medium (MM9) of the chrome-azurol-S (CAS) agar (44). Total iron content of the fish juice was determined according to the AOAC phenanthroline method using standard addition and was estimated to be 1.8 μM. Agar diffusion assay for inhibitory activity against other bacteria was done by using the well diffusion assay in modified IA (MIA) as described earlier (17). MIA supplemented with ethylene-diamine-di(o)-hydroxyphenyl acetic acid (EDDHA; Sigma E-4135) was used for cross-feeding experiments.

Siderophore determination. The spectrophotometric CAS assay (44) was used to determine siderophore production in sterile filtered supernatants of cultures grown in minimal media (CM9 and MM9) or heat-sterilized fish juice under different iron availability conditions (with or without 100 μM $FeCl_3$). Growth was assessed by A_{600}. Arnow's test (3) was used to test for catechol-phenolate-type siderophores, and the hydroxamate type was assayed by the Csáky test (12). Reaction with iron-perchlorate under acid conditions was tested as described by Atkin et al. (4), and the sample was scanned from 400 to 550 nm.

Growth under iron-restricted conditions (with EDDHA). Strains were grown in MIB with 5, 20, 200, 600, 1,000, or 2,000 μM EDDHA. EDDHA was added before autoclaving, and the media were left for at least 48 h at 5°C to secure iron chelation. Growth was determined by A_{600} measurements. All trials were done in duplicate. Growth was recorded after 24 h, and the levels of EDDHA depriving the cultures of iron to an extent at which growth was arrested were determined (6).

Cross-feeding. Ten *S. putrefaciens* strains (five CAS-positive and five CAS-negative strains) were inoculated in MIA containing 20 or 1,000 μM EDDHA. The concentration of EDDHA depended on the concentration of EDDHA arresting growth of the particular strain. Sterilized filter disks (6 mm) were inoculated with 20 μl of each of the same 10 strains and one strain each of *P. fluorescens*, *P. aeruginosa*, and *A. salmonicida*. The disks were placed on the agar, and plates were incubated at 25°C. A sterile filtered solution of $FeCl_3$ served as a positive control. Zones of increased growth of the bacteria inoculated in the agar were read. Some of the surface inoculants were inhibiting growth of the strain in the agar, and in these cases zones of inhibition were read.

Outer membrane proteins. Cultures were grown in heat-sterilized fish juice (with or without 100 μM $FeCl_3$) at 25°C at 150 rpm and harvested after 3 days by centrifugation (16,300 × *g* for 10 min). Pellets were washed and sonicated, and whole cells were removed by centrifugation. The pellet was treated with 2% sarcosyl to dissolve the inner membrane (15), and outer membrane fragments were collected at 48,200 × *g* for 40 min at 4°C. Three units of aprotinin per ml was added as a protease inhibitor (43). Protein was determined by the Lowry method with bovine serum albumin as a standard, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described by Laemmli (25) by using a 5% stacking gel and a 10% separation gel. Samples of 25 to 35 μg of protein were loaded per well. Gels were stained with Coomassie brilliant blue R250.

RESULTS

Siderophore production. More than half (30 of 51) of the *S. putrefaciens* strains assayed produced siderophores in heat-sterilized fish juice as determined by the spectrophotometric CAS assay. Supernatants from 24 strains gave an optical density at 630 nm (OD_{630}) in the CAS assay below −0.9. As the spectrophotometric CAS assay measures the removal of Fe(III) from the blue CAS-Fe(III)-HDTMA complex (43), a negative value at 630 nm indicates a positive siderophore reaction. Six strains were only weakly positive, with OD_{630} ranging from −0.4 to −0.9 (Tables 1 and 2), and did not test positive in the Csáky test. The majority (seven of eight) of strains isolated from aquatic sources were siderophore producing, as were all (six) strains isolated from mesophilic environments (flamingos, wounds, and infections). For the remaining strains, the ability to produce siderophores did not vary systematically with the origin of the strains (Table 1).

S. putrefaciens strains grew on the basal medium of the CAS

TABLE 2. Siderophore production of *S. putrefaciens* strains in heat-sterilized fish juice and growth inhibition with EDDHA in MIB at 25°C

OD_{630} (CAS)[a]	Hydroxyamate[b]	No. of strains[c] with growth arrested at the following concn (μM) of EDDHA:						Total (51)
		>2,000 (25)	2,000 (9)	1,000 (6)	600 (3)	200 (1)	20 (7)	
<−0.9	+[d]	16	5	3	0	0	0	24
−0.4 to −0.9	−	3	1	1	0	1	0	6
>−0.3	−	6	3	2	3	0	7	21

[a] The CAS assay measures the removal of Fe from a blue Fe complex, and therefore, a negative OD at the absorption maximum indicates a positive reaction.
[b] Assayed by the Csáky test (12).
[c] Totals are given in parentheses.
[d] One strain was negative.

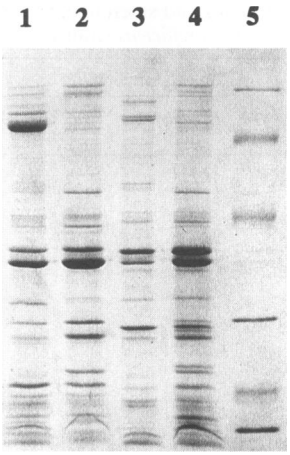

FIG. 1. OMPs of two strains of *S. putrefaciens* grown in heat-sterilized fish juice with (100 μM $FeCl_3$) or without iron. A2 is a siderophore producer, and A11 is negative in the CAS assay. Lanes: 1, A2 with no Fe; 2, A2 plus Fe; 3, A11 with no Fe; 4, A11 plus Fe; 5, standards of 97.4, 66.2, 45, 31, 21.5, and 14.4 kDa.

agar but were unable to grow on the CAS agar when the dye complex was added. With the spectrophotometric version, siderophores were not detected in the liquid basal medium.

All strains were negative in Arnow's test (3) for catechols, but 23 of the 24 strains with strong positive reactions in the CAS assay (OD_{630} < −0.9) were positive in the Csáky test (Table 2).

Iron deprivation with EDDHA. The strains of *S. putrefaciens* differed in ability to grow when iron was chelated with EDDHA (Table 2). The majority (25 strains) of the siderophore producers grew in high (>1,000 μM) concentrations of EDDHA, whereas the seven strains that were inhibited by 20 μM did not produce siderophores. However, several strains that tested negative by the CAS assay were also capable of growth in high concentrations of the iron chelator (Table 2).

Antibacterial action. None of the *S. putrefaciens* strains were capable of inhibiting growth of any of the target organisms (*L. monocytogenes*, *S. aureus*, *A. sobria*, *P. fluorescens*, or *E. coli*) in well diffusion assays in MIA.

Cross-feeding. The addition of EDDHA markedly reduced, but did not completely inhibit, the growth of the strains inoculated in the agar compared with growth in agar with no iron restriction. Several of the siderophore-producing *S. putrefaciens* strains were able to utilize iron chelators from other *S. putrefaciens* strains, and all *S. putrefaciens* strains used compounds produced by the *A. salmonicida* strain under iron-limited conditions (Table 3). A number of strains, mostly the siderophore-negative ones, did not affect the growth of the strain inoculated in the agar. This was seen as the lack of appearance of clearing or growth-intensive zones. Compounds produced by *P. fluorescens* and *P. aeruginosa* under iron-limited conditions did not support growth of *S. putrefaciens* but caused total inhibition of *S. putrefaciens* (seen as clearing zones around the paper disk). Also, growth of siderophore-negative *S. putrefaciens* was inhibited by several siderophore-positive strains (Table 3).

OMPs. The outer membrane protein (OMP) profiles of strains testing positive and negative in the CAS assay are shown in Fig. 1. Both strains were isolated from spoiled fish. When grown under iron-limited conditions, e.g., in fish juice, the siderophore-positive strains produced one new major OMP of approximately 72 kDa. The siderophore-negative strains also showed two weak bands between 75 and 80 kDa,

TABLE 3. Siderophore cross-feeding or growth inhibition of *S. putrefaciens* strains under iron-limited conditions

Surface inoculum[a]	Siderophore production[b]	Growth for MIA inoculum[c]:									
		A2	ATCC 8071	NCTC 10735	A111	A144	A11	A38	A101	A107	A143
A2	+		o	o	o	o	−	−	−	−	−
ATCC 8071	+	o		+	+	+	−	−	−	−	−
NCTC 10735	+	o	o		+	+	−	−	−	−	−
A111	+	−	o	+		+	−	o	−	−	o
A144	+	o	+	+	+		−	−	−	−	−
A11	−	o	o	o	o	o		o	+	o	o
A38	−	o	o	o	o	o	o		+	o	o
A101	−	o	o	o	o	o	o	o		o	o
A107	−	o	o	o	o	o	o	o	+		o
A143	−	o	o	o	o	o	o	o	+	o	
P. fluorescens	+	−	−	−	−	−	−	−	−	−	−
P. aeruginosa	+	−	−	−	−	−	−	−	−	−	−
A. salmonicida	(+)	+	+	+	+	+	+	+	+	+	+

[a] A2, A11, A38, A101, A107, and A111 were isolated from fresh and spoiled fish; A143 and A144 were isolated from water.
[b] CAS reaction after growth for 4 days in heat-sterilized fish juice at 25°C and 150 rpm. (+), OD_{630} (CAS) = −0.45.
[c] For A11, A101, and A107, 20 μM EDDHA was used; for all other strains the concentration was 1,000 μM. +, enhanced; −, inhibited; o, unaffected.

but the strong band of 72 kDa did not appear under iron-limited conditions (Fig. 1). Two proteins of approximately 53 and 23 kDa were seen for both strains when iron was in surplus.

DISCUSSION

S. putrefaciens requires a respiratory metabolism and is able to use a large number of compounds as electron acceptors including ferric iron (14). Its iron requirement under aerobic conditions has, to my knowledge, not been studied, and the present study demonstrates that many strains of *S. putrefaciens* produce siderophores under aerobic and iron-limited conditions.

The ability to produce siderophores was seen with strains from many different environments (Table 2), and thus, there does not seem to be any particular niche in which this ability is especially favorable. It is interesting that NCTC 10735, which is isolated from oil brine, where iron must be expected to be in surplus, is also siderophore positive.

The iron-chelating capability favored growth under iron-restricted conditions (Table 2). However, some isolates grew in high concentrations of EDDHA without testing positive in the CAS assay. A similar behavior has been found for other bacteria like *A. salmonicida* and has been attributed to a siderophore-independent iron acquisition mechanism (22). Although growth was supported by several minimal media (CM9 and MM9), neither allowed production of siderophores, and the fish juice proved superior in this respect. It is well known that siderophore production is medium dependent (30), and the minimal media may lack factors necessary for siderophore production. Since the basal medium of the CAS agar did not support siderophore production, *S. putrefaciens* strains were unable to grow on the agar when iron was chelated by CAS. Schwyn and Neilands (44) reported that the dye complex could be toxic to some bacterial species; however, all strains of *S. putrefaciens* grew on an iron-rich agar with the dye added.

Siderophores of the phenolate type are very common in gram-negative bacteria like strains of the *Enterobacteriaceae* (6, 40, 42), *Vibrio* spp., (2), and aeromonads (22, 31). However, hydroxamates also are produced by several gram-negative bacterial species, like *Salmonella* spp. (6), *V. anguillarum* (30), and *Vibrio cholerae* (1). In the present study, *S. putrefaciens* tested negative for the catechol type, and the *S. putrefaciens* strains with a strong reaction in the CAS assay gave a positive reaction in the Csáky test (Table 2).

Most of the strains with a pronounced CAS reaction and a positive Csáky test had a red or orange color in iron-perchlorate (4). Scanning of the reaction mixture gave flat-top curves with maxima around 480 to 495 nm (data not shown). Although this assay is rather unspecific (35), the flat curve, according to Atkin et al. (4), indicates that a mixture of chelating hydroxamate compounds was produced.

The hydroxamate bidentate has a lower affinity for iron than the phenolates (21, 39), and none of the *S. putrefaciens* strains were capable of inhibiting growth of other species under iron-limited conditions. In contrast, *Pseudomonas* spp. which produce siderophores including the catechol-binding group have a strong inhibitory effect on other microorganisms (17). Also, some *Pseudomonas* spp. produce other antimicrobial substances under iron-limited conditions (45). It was suggested that inhibition of *S. putrefaciens* strains by strains of *Pseudomonas* spp. was caused by iron competition and that this may give *Pseudomonas* spp. a selective advantage when they are growing on or in certain fish products (17). Despite the iron-chelating abilities of *S. putrefaciens*, its siderophores do not seem to give it any advantage vis-à-vis *Pseudomonas* spp. Fish substrates appear to be excellent media for siderophore production, and *Pseudomonas* spp. that produce high-affinity siderophores can, thus, be expected to dominate the microflora of chilled fish.

Siderophores have been extracted from soil (36) and infected fish (30), but except for a study of blue cheese (38), the present study is the first to demonstrate the formation of siderophores in a medium based on food substrates. Free iron occurs only in low concentrations in several foods, and siderophore-mediated interaction may be important for the selection of a microflora in several food products.

It has recently been demonstrated that the ferric reductase activity of *S. putrefaciens* grown under anaerobic iron-rich conditions is associated with the outer membrane (32). This study demonstrates that, under aerobic, iron-rich conditions, at least two proteins appear in the outer membrane (Fig. 1). It is not known whether they are enzymes dependent on iron in their structure or whether they are in any way linked with the uptake of iron by the cell.

In gram-negative siderophore-producing bacteria, iron limitation also induces the formation of new OMPs which are believed to be the receptors for the siderophore-iron complex (23). The size range of these OMPs is 62 to 77 kDa for *V. cholerae* (46), 76 to 83 kDa for *A. salmonicida* (9), and 79 and 86 kDa for *V. anguillarum* (11). Thus, the new OMP of approximately 72 kDa found in siderophore-positive *S. putrefaciens* under iron-limited conditions is comparable in size to the OMPs induced by iron limitation in other bacteria. It is thus likely that the 72-kDa protein is the siderophore receptor of this species.

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