BioMetals **17:** 471–481, 2004.

The structure of salmochelins: *C*-glucosylated enterobactins of *Salmonella enterica*[§]

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Received 31 January 2004; Accepted 5 February 2004; Published online: April 2004

Key words: Siderophores, salmochelins, enterobactin, Salmonella enterica, *Escherichia coli*

Abstract

Salmochelins represent novel carbohydrate containing catecholate siderophores, which are excreted by *Salmonella enterica* and uropathogenic *Escherichia coli* strains under low-iron stress. While previous analytical data showed salmochelins to contain 2,3-dihydroxybenzoyl-L-serine and glucose, the molecular structure remained elusive. Structure elucidation with electrospray ionization-Fourier transform ion cyclotron resonance-mass spectrometry (ESI-FTICR-MS), GC-MS and 2D-NMR now revealed that salmochelins are enterobactin-related compounds, which are β-*C*-glucosylated at the 5-position of a 2,3-dihydroxybenzoyl residue. The key compound salmochelin S4 is a twofold β-*C*-glucosylated enterobactin analogue. Comparison of partial structures of salmochelin with a *C*-glycosylated compound previously characterized by another group strongly suggest that salmochelins represent the long sought compounds termed Salmonella resistance factors (SRF) or pacifarins. Transformation of *iro*-genes into enterobactin-producing *E. coli* K12 confers the ability to produce salmochelins. A detailed analysis proved *iroB* to be the sole gene with glycosyltransferase activity necessary for salmochelin production. Salmochelins compared to enterobactin are the better siderophores in the presence of serum albumin. This may indicate for salmochelins a considerably more important role for pathogenic processes in certain *Escherichia coli* and *Salmonella* infections than formerly assigned to enterobactin. This conclusion is supported by the location of the *iro* genes on pathogenicity islands of uropathogenic *E. coli* strains.

Introduction

Siderophores are microbial ferric-specific chelators excreted under low-iron conditions to sequester iron essential for growth in aerobic microorganisms. A considerable number of siderophores have been described, containing mainly catecholate, hydroxamate and carboxylate oxygen donor groups which contribute to the high binding affinity for Fe^{3+}. The most prominent member among the catecholate siderophores is enterobactin (also named enterochelin) (Figure 1) isolated from *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (Pollack & Neilands 1970; O'Brien & Gibson 1970). Enterobactin is predisposed to iron binding and has the highest stability constant of all known siderophores (Raymond *et al.* 2003).

Iron uptake via siderophores is an energy-requiring membrane-receptor dependent process which has been analyzed in detail in gram-negative and gram-positive bacteria (Braun & Hantke 2001). Siderophore biosynthesis has been regarded as an important virulence factor in pathogenic processes of bacterial infections in order to enable iron-supply in serum environment (Braun 2001; Weinberg 1978). Depriving microorganisms of iron supply prevents growth and invasion of

[§]This article is dedicated to the memory of Igor Stojiljkovic who helped to find the *iro* genes in 1996 by his Fur titration assay.

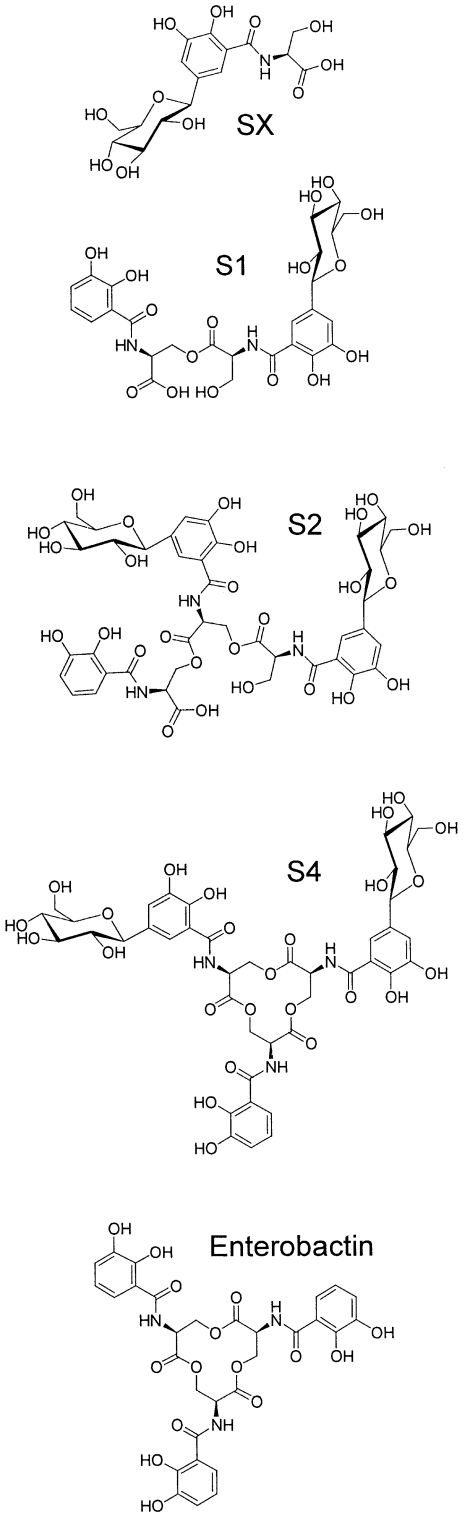

Fig. 1. Structure formulas of enterobactin and the salmochelins SX, S1, S2 and S4. Salmochelin SX is identical to parcifarinic acid (Fu 1985).

host tissues. Although enterobactin has always been thought to be essential for the growth of *E. coli* and *Salmonella*, its role in pathogenesis has been questioned due to its binding to hydrophobic sites of serum albumin (Konopka & Neilands 1984). Recent studies indicate that lipocalin NGAL additionally contributes to the bacteriostatic effect of serum by tightly binding enterobactin (Goetz *et al.* 2002). In addition, *Salmonella* mutants deficient in enterobactin biosynthesis and uptake were attenuated in some infection models (Gorbacheva *et al.* 2001) while they stayed fully virulent in others (Erikson *et al.* 2003, Tsolis *et al.* 1996). It seems that only certain steps during the infection process depend on siderophore iron supply by the enterobactin/salmochelin system. In the gut, where most *Salmonella* infections start, iron supply strongly depends on the actual diet and thus only in an iron poor diet would siderophore production be necessary for infection of the host.

In a recent study we described novel catecholate siderophores named salmochelins, which are the major siderophores from *S. enterica* and certain *E. coli* strains (Hantke *et al.* 2003). While enterobactin could be easily extracted into ethyl acetate, salmochelins remained in the aqueous phase. A first analytical characterization of salmochelins revealed 2,3-dihydroxybenzoic acid (DHB) and L-serine as major constituents (Hantke *et al.* 2003) suggesting structural similarities to enterobactin from *S. enterica*. We report here on the structural characterization (FTICR-MS, GC-MS, 2D-NMR) of salmochelins SX, S1, S2 and S4, all of which contain *C*-glycosidically bound glucose to C5 of 2,3-dihydroxybenzoic acid moieties.

Materials and methods

Strains and growth conditions

The strains and plasmids used are described in Table 1. Strains were routinely grown on TY medium containing (per liter): tryptone (8 g), yeast extract (5 g), and sodium chloride (5 g). Minimal medium M63 was previously described (Hantke *et al.* 2003). NBA-agar for growth tests in the presence of albumin contained (per liter) 2.5 g NaCl, 4 g trypton, 6 g agar, 150 μM dipyridyl, 150 μM ethylenediamine-*bis*-(*o*-hydroxyphenylacetic acid), 0.2% glucose. About 1×10^8 cells were added per plate to the agar before pouring. Where indicated, 50 g bovine serum albumin per liter was added to the agar. Filter paper discs

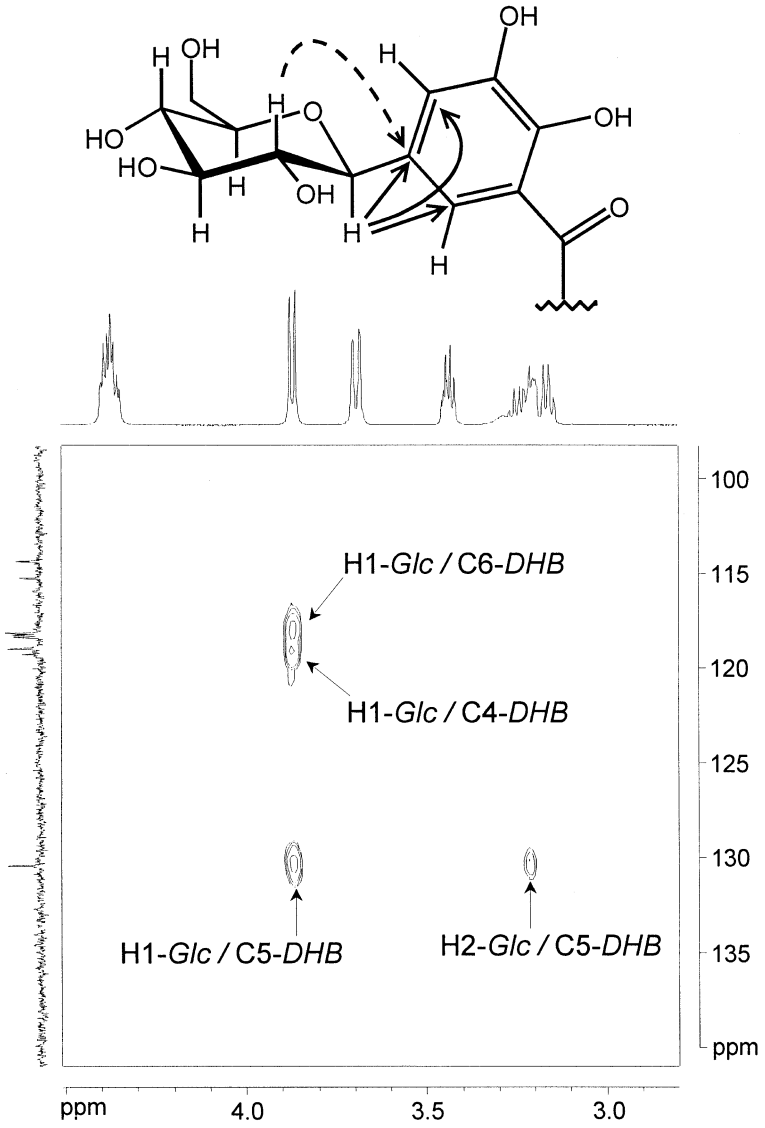

Fig. 2. Part of the HMBC-NMR spectrum of Salmochelin S4. Arrows (above) indicate correlations from the HBMC-spectrum (below).

loaded with 10 μl (1 mg/ml) of siderophore solution were placed on the agar plates and growth zones were scored after 18 h.

Construction of plasmids

Plasmid pKHI18 was obtained by digesting plasmid p332 (Sorsa *et al.* 2003) with *Hin*dIII and ligating the insert into the *Hin*dIII digested low copy number vector pWSK29 (Wang & Kushner 1991).The sequence of the *iro* genes may be found in the genebank under the accession number AY205565. pKHI19 was obtained by a *Nhe*I/*Bln*I digest of pKHI18, making the sticky ends blunt with Klenow enzyme and religation. This led to a deletion of *iroED* and half of *iroC*. pKHI20 was obtained by a *Xba*I/*Bln*I digest of pKHI18 and religation of the blunted ends. This led to a deletion of *iroN* and the C-terminal part of *iroE*. pKHI21 was obtained by *Bst*BI digest of pKHI18 and religation. This led to a deletion of the N-terminal part of *iroN*.

Isolation of salmochelins

Salmochelins (SX, S0, S1, S2 and S3) were enriched from 6 $\times$ 1-liter batch cultures of *S. enterica* serotype Paratyphi B IHS1319, while S4 was isol-

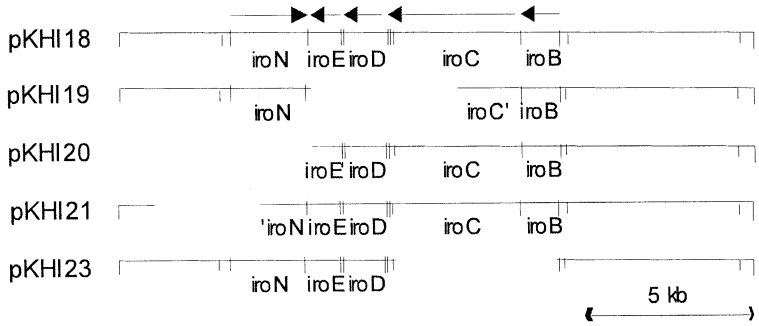

Fig. 3. Map of the *iro* genes on plasmid pKHI18 and the deletions in the derived plasmids.

Table 1. List of strains.

Strain designation / Relevant genotype		**Reference**
Escherichia coli strains		
E. coli K-12 MG1655		Lab collection
E. coli 635 ΔPAI III	*iroNEDCB*	(Dobrindt *et al.* 2001)
Salmonella enterica strains		
Paratyphi B IHS1319		(Hantke *et al.* 2003)
Stanleyville 207/81		(Hantke *et al.* 2003)
H5547	*aroA*$^+$ *iroC*::kan	(Hantke *et al.* 2003)
Plasmids		
p332	*iroNECDB*	(Sorsa *et al.* 2003)
pKHI18	*iroNEDCB*	This work
pKHI19	*iroNB*	This work
pKHI20	*iroDCB*	This work
pKHI21	*iroEDCB*	This work
pKHI23	*iroNED*	This work

ated from 10 × 1-liter batch cultures of *S. enterica* H5547. The siderophores were separated by DEAE cellulose column chromatography as previously described (Hantke *et al.* 2003). Crude salmochelin fractions (20 mg) were chromatographed on a prepLCMS Merck-Hitachi HTP-MS System (Merck, Darmstadt, Germany) using a Purospher-STAR RP-18e column (5 μm, 25 × 100 mm, Merck, Darmstadt, Germany). Solvent A was 0.1 % Trifluoroacetic acid (TFA) and solvent B was acetonitrile/0.1% TFA (gradient 5–40% over 20 min with a flow rate of 15 ml/min). Detection was performed by ESI-ion trap-MS (Merck-Hitachi M-8000, Merck, Darmstadt, Germany) and multiwave UV-vis detection (Merck-Hitachi L-7410, Merck, Darmstadt, Germany). Coeluting salmochelins were rechromatographed to analytical purity (>95%).

Mass spectrometry and 2D NMR experiments

LC-MS experiments were performed on an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) coupled to a Bruker Esquire 3000+ (Bruker-Daltonics, Bremen, Germany). FTICR-ESI-MS spectra were recorded on an APEX II FTICR mass spectrometer (4.7 T, Bruker-Daltonics, Bremen, Germany). To enhance mass accuracy, spectra were measured with internal mass standards. NMR spectra were recorded on an AMX 600 NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a 5 mm triple-resonance probehead with z-gradients. GC-MS experiments were performed with an Agilent HP5973/6890 instrument.

GC-MS experiments

Chiral and quantitative amino acid analysis: Salmochelin samples were hydrolyzed (24 h/110 °C/6N HCl), derivatized (OEt/trifluoroacetic acid) and subsequently analyzed by GC-MS using a Chirasil-Val capillary. Quantitative analysis was performed by enantiomer labelling (Frank *et al.* 1978). Dihydroxybenzoic acid: Prior to amino acid analysis, hydrolysates were saturated with NaCl and extracted with ethyl acetate. The organic phase was evaporated to dryness and derivatized with 1:1 BSTFA in acetonitrile (60 °C/1 h). The products were analyzed by GC-MS on a DB-5 capillary. Methanolysis: Salmochelin samples were heated at 110 °C for periods of between 15 min and 17 h in 2N HCl in dry methanol. After removal of excess reagent, *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA)/acetonitrile (1:1) was added and heated at 60 °C for 1 h. Once again, the products were analyzed by GC-MS on a DB-5 capillary.

Table 2. Assignment of molecular masses by high-resolution ESI-FTICR-MS

Salmochelin	Molecular Ion	Accurrate Mass	Molecular Formula	Δ[ppm]
SX	$[M-H]^-_{exp}$	402.10416	$C_{16}H_{21}NO_{11}$	0.05
	$[M-H]^-_{theor}$	402.10418		
S1	$[M-2H]^{2-}_{exp}$	312.07231	$C_{26}H_{30}N_2O_{16}$	0.56
	$[M-2H]^{2-}_{theor}$	312.07249		
S2	$[M-2H]^{2-}_{exp}$	504.62210	$C_{42}H_{49}N_3O_{26}$	1.71
	$[M-2H]^{2-}_{theor}$	504.62294		
S3	$[M+H]^+_{exp}$	1012.26804	$C_{42}H_{49}N_3O_{26}$	0.28
	$[M+H]^+_{theor.}$	1012.26771		
S4	$[M+Na]^+_{exp}$	1016.23924	$C_{42}H_{47}N_3O_{25}$	0.16
	$[M+Na]^+_{theor}$	1016.23908		

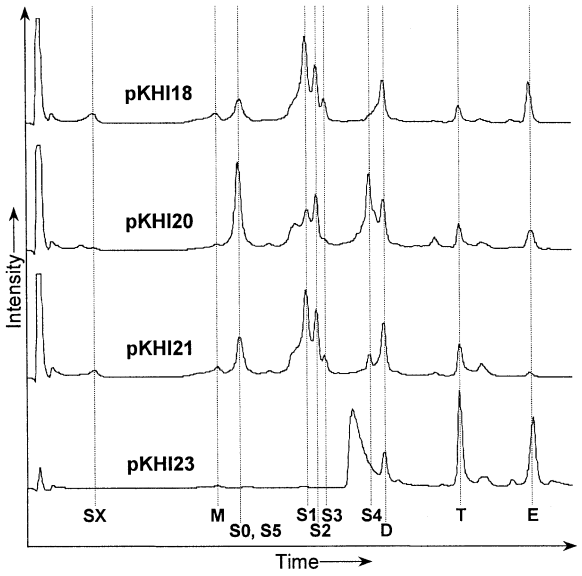

Fig. 4. HPLC analysis of the siderophores produced by *E. coli* K-12 pKHI18 *iroNEDCB*; and *E. coli* K-12 pKHI20 *iroDCB, E. coli* K-12 pKHI21 *iroEDCB*, and *E. coli* K-12 pKHI23 *iroNED*. Vertical lines indicate salmochelins identified by ESI-MS: SX, S0, S1, S2, S3, S4, S5. M, D, T are monomer, dimer and trimer of 2,3-dihydroxybenzoylserine respectively, and E is enterobactin.

Ozonolysis: of salmochelin S2

Ozone was bubbled for 2 h through a solution of salmochelin S2 in methanol cooled with acetone/dry ice (Narayanan & Seshadri 1971). The solution was then taken to dryness and the products derivatized (OMe/TMS) as above.

Data base searches

Stuctures were compared using the Dictionary of Natural Products on CD-ROM, Version 11.2, Chapmann & Hall, London, 2003 and Scifinder®, Version 2002.1, American Chemical Society

Results

Structure elucidation of salmochelins

For isolation of salmochelins in mg-quantities, culture supernatants from *S. enterica* Serotype Paratyphi B IHS1319 or H5547 were applied to DEAE-cellulose ion exchange chromatography as previously described (Hantke *et al.* 2003). The enriched material was analyzed with HPLC, HPLC-ESI-MS and separated with preparative HPLC-ESI-MS as described in materials and methods. Combined UV-vis and mass spectrometric data indicated seven compounds (SX, S0, S1, S2, S3, S4, and S5) as members of the salmochelin family. Molecular formulae of salmochelins were derived from accurate molecular mass determination by high-resolution ESI-FTICR-MS as shown in Table 2. Molecular formulae determined by FTICR-MS for salmochelins revealed increasing numbers of nitrogen atoms SX (1N), S1 (2N), S2/S3 (3N) and S4 (3N). The mass difference of $\Delta m = 18$ Da between salmochelin S2/S3 and S4 suggested a macrolactonization similarly to enterobactins T ($(DHB\text{-}Ser)_3$ linear trimer) and E (enterobactin). The mass difference of 162 Da between putative salmochelin SX and M (DHB-Ser monomer unit of enterobactin) as well as for salmoch-

elin S1 and D (DHB-Ser)$_2$ linear dimer) suggested a $C_6H_{11}O_5$ (hexosyl) modification.

For further structure analysis, samples of purified salmochelins SX, S0, S1, S2 and S4 were hydrolyzed and extracted with ethyl acetate. Upon derivatization, the aqueous (TFA/ethyl esters) and ethyl acetate (TMS derivatives) fractions were analyzed by GC-MS. In the aqueous fraction of these salmochelins, L-serine was present as sole amino acid while 2,3-dihydroxybenzoic acid (DHB) was found in all EtOAc-extracts. Upon methanolysis followed by derivatization, DHB-Ser was identified as the principal constituent together with lesser amounts of DHB (both as the $(TMS)_3$ and the TMS_2/OMe derivatives), the proportion depending on the duration of methanolysis. In addition, minor but significant amounts of glucose in the form of its methyl glycoside/TMS derivative were found. Two peaks could be assigned to DHB-hexose (TMS/methyl ester and TMS/TMS-ester) on the basis of their mass spectra. Other components in the GC-MS-chromatogram indicated the presence of both nitrogen and a carbohydrate moieties; however, assignments of these products were hampered by the limited mass range of the GC-MS (max. *m/z* 800).

While the results of the methanolysis indicated the presence of glucose in salmochelins, its low yield relative to DHB-Ser and the relative hydrolytic stability of the DHB-hexose compound argued against its *O*-glycosidic attachment. To clarify this point, salmochelins were investigated by 2D-NMR (COSY, TOCSY, NOESY, HSQC and HMBC). The interpretation of 2D-NMR spectra was firstly performed with salmochelin S4 (Figures 1 and 2). HMBC-experiments clearly showed, that three serine moieties were linked via ester bonds involving the hydroxy sidechains and the C-terminus to form an enterobactin-like triseryl 12-membered macrocycle. Accordingly, the amino group of each serine was amidated with DHB residues. Moreover, in the spectra characteristic signals derived from two carbohydrate moieties were found. However, ^{1}H- and ^{13}C-chemical shifts (e.g. 3.87 ppm / 81.2 ppm) of the anomeric carbons were shifted to unusually high field. HMBC correlations between the proton of the anomeric carbon of the carbohydrate residue and aromatic ring carbons (C4, C5 and C6) of DHB indicated a *C*-glycosidic bond to the 5-position of the aromatic ring (Figure 2).

Since the identity of the carbohydrate residue could not be unequivocally determined from NMR coupling constants, salmochelin S2 was submitted to ozonolysis (Narayanan & Seshadri 1971). On the basis of their mass spectra and their retention times, two of the products of ozonolysis were identified by GC-MS as glucose and arabinose (TMS/methylglycosides). The mass spectrum of the major product could be interpreted as arising from a heptonic acid (as its TMS/methylester derivative). Comparison of the mass spectrum and the retention time with those of the authentic substance (Dromowicz & Koell 1988) confirmed that it was in fact 2,6-anhydro-D-glycero-D-gulo-heptonic acid. The presence of a β-glycosidic bond, indicated by the coupling constant of $J = 9.0$ Hz from the proton of the anomeric carbon to the proton of C2 of the glycosyl residue was confirmed by the formation of 2,6-anhydro-D-glycero-D-gulo-heptonic acid, with its equatorial carboxy group upon ozonolysis of salmochelin S2. Comparison of retention times on the chiral stationary phase (Lipodex A) of authentic D- and L-glucose anomers (as TFA/methyl glycoside derivatives) with those of the ozonolysis products of salmochelin S2 indicated that salmochelin-bound glucose possesses the D-configuration. The same type of glycosylation with glucose was assumed for all other above mentioned salmochelins.

In summary salmochelin S4 is a twofold β-*C*-glucosylated enterobactin analogue. Accordingly, NMR-analysis revealed salmochelin S2 as the linearized hydrolysis product of salmochelin S4 bearing one unglycosylated DHB-serine moiety located at the C-terminal end. Furthermore, salmochelin S1 is a dimer with DBH(glucosyl)-seryl-DHB-serine constitution and salmochelin SX is the monomeric DHB(glucosyl)-serine molecule. The structures of salmochelins S3 and S5 could not be determined with certainty because there was insufficient material available for NMR-characterization. Since salmochelins S2 and S3 have the same exact molecular mass, S3 is presumed to be an isomer. According to the molecular mass of $[M+H]^+_{exp} = 789.2$, salmochelin S5 is concluded to be the dimer of salmochelin SX. The molecular mass of $[M-H]^-_{exp} = 1016.19644$ for salmochelin S0 could not be assigned to any reasonable structure. However, upon dissolving in salt-free solvents it rapidly converted into salmochelin S4. In analogy to enterobactin containing isolates, which are also accompanied by linear mono-, di-, and trimeric degradation products similar oligomeric structures are found for salmochelins with S4 as the key metabolite. Remarkably, threefold glycosylated salmochelins have not been detected with LC-ESI-MS. The structural formulae of salmochelins SX, S1, S2, and S4 determined

by NMR-experiments are summarized in Figure 1 and the NMR-data of salmochelin S4 are shown in Table 3. NMR-data of salmochelin S1 and S2 are available as Supporting Information (data not schown).

The iro genes allow synthesis of salmochelins by E. coli *K-12*

We recently showed that the production of salmochelins is dependent on the *iro* genes (Hantke *et al.* 2003). A mutant in *iroB* was unable to produce salmochelins and in these cultures only enterobactin and its degradation products (monomer (M), dimer (D) and trimer (T) of 2,3-dihydroxybenzoylserine) were found. HPLC-analyses of culture filtrates of the uropathogenic strain *E. coli* 536 revealed salmochelins (data not shown) as expected since the *iro* gene cluster is localized on the pathogenicity island III (PAI3) (Dobrindt *et al.* 2001). In contrast, mutant 536 ΔPAI3 with a deleted pathogenicity island retained only the ability to produce enterobactin. This mutant was transformed with the plasmid pKHI18 containing the *iroBCDEN* gene cluster which was cloned from p332 (Sorsa *et al*. 2003) into a low copy number vector (Figure 3). As revealed by HPLC-ESI-MS analysis (Figure 4) pKHI18 restored the ability to produce salmochelins indicating that the plasmid contained a functional *iro* gene cluster. *E. coli* K-12 MG 1655, unable to produce salmochelin, was transformed with pKHI18 *iroBCDEN* and was grown in low-iron glycerol minimal medium (M63). After 18 hours cells were sedimented and siderophores were isolated from the culture supernatant. HPLC analysis revealed that roughly 80% of the siderophores were salmochelins (SX–S5) while enterobactin and its hydrolysis-derived products (M, D, T) made up only 15% of the siderophores detected. These findings indicated that the enterobactin biosynthesis machinery of *E. coli* K-12 is able to collaborate with the Iro proteins to produce salmochelins.

Only iroB is necessary for salmochelin production

In order to further characterize the impact of the *iro* genes on the synthesis of the salmochelins, various plasmid constructs were transformed into *E. coli* K-12. The strain with plasmid pKHI21 *iroECDB* lacking *iroN* (Figure 3) which encodes the outer membrane salmochelin receptor, had a slightly altered salmochelin production pattern. Besides enterobactin compounds the HPLC-chromatogram (Figure 4) showed

Table 3. ^{1}H and ^{13}C-NMR data of salmochelin S4 in DMSO-[D6].

Position		^{1}H/δ [ppm]	^{13}C/δ [ppm]
Ser-1	1	–	169.4
	2	4.89	50.9
	3	4.36	63.0
		4.67	
	NH	9.14	–
DHB-1	CO	–	168.8
	1	–	115.4
	2	–	148.1
	3	–	145.4
	4	6.93	118.8
	5	–	130.3
	6	7.35	117.9
	2-OH	11.56	–
	3-OH	9.30	–
Glc-1	1	3.87	81.2
	2	3.21	74.0
	3	3.25	78.1
	4	3.16	70.3
	5	3.20	81.0
	6	3.43	61.2
		3.69	
Ser-2	1	–	169.4
	2	4.89	50.9
	3	4.36	63.0
		4.67	
	NH	9.14	–
DHB-2	CO	–	168.8
	1	–	115.4
	2	–	148.1
	3	–	145.4
	4	6.93	118.8
	5	–	130.3
	6	7.35	117.9
	2-OH	11.56	–
	3-OH	9.30	–
Glc-2	1	3.87	81.2
	2	3.21	74.0
	3	3.25	78.1
	4	3.16	70.3
	5	3.20	81.0
	6	3.43	61.2
		3.69	
Ser-3	1	–	169.3
	2	4.91	50.9
	3	4.38	63.0
		4.63	
	NH	9.08	–
DHB-3	CO	–	168.8
	1	–	115.3
	2	–	149.8
	3	–	146.1
	4	6.95	118.8
	5	6.74	118.2
	6	7.35	117.9
	2-OH	11.60	–
	3-OH	9.33	–

an increased production of cyclic salmochelin S4 compared to *E. coli* K-12 pKHI18.

Strain *E. coli* K-12 pKHI20 *iroBCD* lacking *iroN* and *iroE*, the latter encoding a putative periplasmic hydrolase, produced increased amounts of cyclic salmochelin S4 and reduced amounts of salmochelins (S1/S2) as well as enterobactin and its hydrolysis products (Figure 4). *E. coli* K-12 pKHI19 *iroNB* produced neither enterobactin nor salmochelins (data not shown), which may be explained by the deletion of the C-terminal end of IroC preventing export of S4 via the putative IroC export protein. Accumulation of S4 inside the cell may be the reason for an inhibited secretion of enterobactin. Also uropathogenic *E. coli* 536 ΔPAI3 pKHI19 formed very low amounts of salmochelin S4 and enterobactin together with their hydrolysis products and the linear salmochelins S1 and S2 were not detected. *E. coli* K-12 pKHI23 deficient in IroB and IroC (Figure 3) produced only enterobactin and its linear degradation products. These experiments confirm that neither IroD, which is expected to be a cytoplasmic esterase nor IroE are involved in the biosynthesis of salmochelin S4 (Figure 4). The linear degradation products S1 and S2 occur only in appreciable amounts when IroE and IroD are present. Previous data support these findings, since *S. enterica* serotype Typhimurium AIR50 with a chromosomal *iroD::*kan mutation produced mainly S4 (Hantke *et al.* 2003). This strain is unable to utilize S4 or S2 as an iron source which may indicate that IroD is necessary for iron removal, similar to its homolog, the enterobactin esterase Fes (Porra *et al.* 1972; Winkelmann *et al.* 1994).

Salmochelin is a better siderophore than enterobactin in the presence of albumin

It had been shown earlier that binding of enterobactin to serum albumin (Konopka & Neilands 1984) and NGAL lipocalin (Goetz *et al*. 2002) may severely impair its role as an iron provider for bacteria in serum. Since salmochelins possess a more hydrophilic nature compared to enterobactin (Hantke *et al.* 2003), we studied iron supply via salmochelins and enterobactin in growth promotion tests in the presence of albumin. On iron-limited agar medium, *S. enterica* serotype Stanleyville showed a growth zone (ø 18 mm) around a filter paper disc which was reduced to (ø 11 mm) in the presence of serum albumin. The same assay performed with salmochelins S4 and S2 without (ø 24 - 23 mm) and with serum albumin (ø 20 - 19 mm) resulted in a significantly smaller growth reduction effect (Table 4). This indicated that salmochelin S4 and S2 are much better iron sources than enterobactin in the presence of albumin. In addition, the salmochelins seem to have better diffusion properties in the agar medium than enterobactin.

Table 4. Siderophore dependent growth of *S. enterica* subspecies Stanleyville on iron restricted nutrient broth agar (NBA) plates. Filter paper discs (6 mm diameter) were loaded with siderophores (10 μg) and the growth zone was read after 24 h of incubation at 37 °C.

	Diameter of growth zone (mm)	
	NBA	NBA + 50 mg albumin/ml
Salmochelin S4	24	20
Salmochelin S2	23	19
Enterobactin	18	11

Discussion

Salmochelins and pacifarins

Database searches on the structure of salmochelin S0-S4 revealed no structures identical to the salmochelins S1, S2 and S4 but the monomeric structure of salmochelin SX was contained in a thesis from 1985 (Fu 1985) there designated pacifarinic acid. However, as the author stated, pacifarinic acid was not biologically active and an oligomeric structure was explicitly excluded.

The work on pacifarins goes back to H.A. Schneider who began in 1940 investigating the effects of foodstuffs on Salmonella infections (Schneider 1967; Wawszkiewicz 1975). Two groups of mice were fed with a natural and a semisynthetic diet. Upon infection with a combination of avirulent and virulent *S. typhimurium* strains, mice fed with the semisynthetic diet died whereas most animals on the natural diet survived. Vitamins and antibiotics as protective cause in the natural diet were excluded. Prevention of killing was attributed to a *Salmonella* resistance factor (SRF), also called 'pacifarin' from the Latin verb *pacificare*, to pacify. The *Salmonella* resistance factor was first believed to be a component of foodstuffs e.g. corn, rye, rice, tea, etc. and finally egg-white powder. Detailed investigation of the latter revealed that in fact bacteria such as *Enterobacter cloacae* were the true source of pacifarin. An elevated pacifarin production was

found under low-iron growth conditions, indicating a relationship to siderophores. From these cultures two active fractions conferring pacifarin activity were isolated, an ethyl acetate extractable and a watersoluble fraction both containing catechols and serine.

However, with the isolation of ethyl acetate extractable enterobactin from *S. typhimurium* strains by Pollack and Neilands (1970) displaying comparable pacifarin activity (Wawszkiewicz *et al*. 1971), further interest in the water soluble pacifarins waned, leaving the chemical structures of these compounds unsolved. Subsequent research focussed solely on enterobactin, which is nowadays a prime example for the biosynthesis of catecholate siderophores (Raymond *et al.* 2003; Roche &Walsh 2003). Much later the thesis of J.M. Fu (1985) described pacifarinic acid to be a constituent of one of the water soluble pacifarin fractions from the Schneider group (Wawszkiewicz 1975) which is identical to salmochelin SX. We therefore suppose, that herein presented salmochelins are the long sought pacifarins described by Schneider.

Our results show that salmochelins S1, S2, S4 and dihydroxybenzoyl serine are taken up mainly by the IroN outer membrane receptor of *S. enterica*. Enterobactin is not considered as a major virulence factor in *Salmonella* infections (Konopka & Neilands 1984; Raymond *et al*. 2003) but salmochelins may enhance virulence. This is also supported by several reports on the expression of the *iro* genes under iron poor conditions (Bäumler *et al.* 1996; Bjarnason *et al.* 2003) as they prevail in serum and under certain pathogenic situations (Dozois *et al.* 2003). It seems that only certain steps during the whole infection process depend on siderophore-mediated iron supply by the enterobactin/salmochelin system. Most *Salmonella* infections start in the gut where siderophore production strongly depends on the actual diet, which may contain widely varying amounts of available iron. This may also be the reason for the controversial discussion on the role of enterobactin (as we now know salmochelin) in *Salmonella* infections (see discussion in Rabsch *et al.* 2003).

The effect of pacifarins still awaits an explanation. In serum low-iron growth conditions prevail, since in a healthy person only about 30 % of serum transferrin is saturated. These iron-deficient conditions may then cause the expression of certain virulence factors in *Salmonella* needed for the next step in the infection process. The presence of salmochelins may improve iron supply of *Salmonella* under these conditions thereby leading to a lowered expression of certain virulence factors. For instance the iron regulator Fur influences some genes of the acid response regulation which is important for their survival in lysosomes (Foster 2000). Whether this hypothesis explains the pacifarin effect remains to be shown.

Biosynthesis of salmochelins

The production of salmochelins by *E. coli* K-12 pKHI18 demonstrates that all preconditions for its biosynthesis are found in this laboratory strain. The complementation assays with *E. coli* 536 ΔPAI III pKHI19 *iroNB* indicated that only the glycosyltransferase IroB is necessary to switch enterobactin biosynthesis towards biosynthesis of salmochelin S4.

However, the lack of the putative export protein IroC may be the reason why low amounts of siderophores are found in the medium – and the total lack of siderophores in the supernatant of *E. coli* K-12 pKHI19 *iroNB*. In addition, the absence of the IroN receptor protein in *E. coli* K-12 pKHI21 *iroEDCB* retarded utilization of salmochelin S4 only slightly. In this context, an alternative uptake via other catecholate siderophore receptors seems to suffice for iron supply (Hantke *et al.* 2003). Sequence similarities suggest that the periplasmic IroE protein and the cytoplasmic IroD protein are involved in the hydrolysis of salmochelins. The *E. coli* K-12 pKHI20 *iroDCB* deficient in the expression of IroN and IroE shows an accumulation of S4 in the supernatant and reduced amounts of the linear salmochelins S2 and S1. This indicates that IroE is important for uptake of S4. The mutant *S. enterica* serotype Typhimurium H5547 *iroD*::kan defective in IroD (and possibly in IroE due to polar effects), also accumulated S4 as has been observed (Hantke *et al.* 2003). However, an *E. coli aroB* pKHI18 *iroNEDCB* strain fed with enterobactin, secreted no salmochelins into the supernatant, suggesting that enterobactin inside the cell or its EntF- or EntB-bound precursors may be the substrates for the glycosyltransferase reactions by IroB.

Proteins related next to IroB are glycosyltransferases involved in antibiotic biosynthesis, mainly active as *O*-glycosyltransferases. However, the more distantly related UrdGT2 protein of the urdamycin biosynthesis gene cluster from *Streptomyces fradiae* has been shown to be a *C*-glycosyltransferase (Hoffmeister *et al.* 2003). This indicates that sequence homology is not a sufficient guide to predict the substrate specificity of the enzyme.

In summary we have shown, that salmochelins are C-glucosylated siderophores with salmochelin S4; a twofold *C*-glucosylated enterobactin analogue as the key compound. Salmochelins are suggested to be the long sought pacifarins, which have been presumed to play a major role in *Salmonella* infections. Although a fragment had been isolated earlier named pacifarinic acid, structure elucidation of all other pacifarin compounds has never been published. The interpretation of the pacifarin effects observed by Schneider *in vivo* require further analysis with the now available techniques to study gene expression of salmochelins *in vivo*.

Acknowledgements

We thank Prof. Dr. P. Köll and Dr. A. Lützen (University of Oldenburg, Germany) for providing the 2,6 anhydroaldonic acid standard and Prof. Dr W. A. König (University of Hamburg, Germany) for a gift of perpentyl α-cyclodextrin (Lipodex A), Prof. Dr. G. Jung and Prof. Dr. V. Braun (University of Tübingen, Germany) for helpful discussions. K. Hantke and G.Winkelmann gratefully acknowledge the technical assistance of Kerstin Haible and Sandra Frick. The work of R.D. Süssmuth is supported by an Emmy-Noether Fellowship for young investigators of the Deutsche Forschungsgemeinschaft (DFG) (SU 239/2-2). Further support was granted to K. Hantke (HA 1186/4-2) and R. D. Süssmuth (SU 239/2-1) by the DFG-Forschergruppe.

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