

The colicin G, H and X determinants encode microcins M and H47, which might utilize the catechololate siderophore receptors FepA, Cir, Fiu and IroN

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The colicin G producer *Escherichia coli* CA46, the colicin H producer *E. coli* CA58 and *E. coli* Nissle 1917 (DSM 6601) were shown to produce microcin H47 and the newly described microcin M. Both microcins were exported like colicin V by an RND-type export system, including TolC. The gene cluster encoding microcins H47 and M in strains CA46 and CA58 is nearly identical to that in strain DSM 6601, except that two additional genes are included. A Fur box identified in front of the microcin-encoding genes explained the observed iron regulation of microcin production. The catechololate siderophore receptors Fiu, Cir and FepA from *E. coli* and IroN, Cir and FepA from *Salmonella* were identified as receptors for microcins M, H47 and E492. IroN takes up the glucose-containing catechololate siderophore salmochelin, whose synthesis is encoded in the *iro* gene cluster found in *Salmonella* and certain, often uropathogenic, *E. coli* strains. A gene in this *iro* cluster, *iroB*, which encodes a putative glycosyltransferase, was also found in the microcin H47/M and microcin E492 gene clusters. These microcins could aid the producing strain in competing against enterobacteria that utilize catechololate siderophores.

INTRODUCTION

Escherichia coli Nissle 1917 (DSM 6601, serotype O6:K5:H1), a non-pathogenic faecal isolate (Blum *et al.*, 1995), is the active component of the probiotic Mutaflor®, which is used for the treatment of various gastrointestinal disturbances and diseases (Kruis *et al.*, 1997; Lodinova-Zadnikova *et al.*, 1998; Malchow, 1997; Rembacken *et al.*, 1999). The strain was originally isolated by the physician and bacteriologist A. Nissle during World War I from the only healthy soldier of a group with diarrhoea (Nissle, 1925). Nissle assumed that this *E. coli* strain, which he called Mutaflor, had been responsible for protection of this soldier from infectious diarrhoea and might therefore be useful in sustaining a healthy milieu in the gut by suppressing pathogenic bacteria. *In vitro* studies showed that this particular strain was able to compete with certain *E. coli* strains and other enterobacteria (Papavassiliou, 1959; Nissle, 1925). The strain was shown to produce a bactericidal activity, named colicin X (Papavassiliou, 1959), not to be confused with the colicin X described by Miyama *et al.* (1961), which is now better known as microcin B17 (San Millan *et al.*, 1987). Further characterization of the Mutaflor colicin X showed that it is

active against some *Enterobacteriaceae*, especially against strains of *E. coli* (Papavassiliou, 1961). In addition, colicin X is sensitive to chloroform, which is an unusual property amongst colicins and microcins.

We previously tried to isolate and characterize colicin X and wanted to determine whether the colicin helps the strain to outcompete other strains. However, like Papavassiliou (1961), we were unable to prepare high-activity broth extracts of this colicin, and colicin production was only observed under certain conditions. Since colicins G and H, originally described by Fredericq (1948), showed the same receptor specificity as colicin X, these strains were included in the studies. The isolation of colicins X, G and H was not successful; the molecular biological methods used in the study presented here showed that these colicins are in fact a mixture of two bactericidal activities: microcin H47 and a new microcin, designated microcin M (for Mutaflor).

METHODS

Bacterial strains, plasmids, media and growth conditions.

The bacterial strains used in this study are listed in Table 1. The *Salmonella enterica* serovar Typhimurium LT2 strains TA2700, WR1223, WR1316, WR1330 and WR1332 were kindly supplied by W. Rabsch (Rabsch *et al.*, 1999). Strains H2700, HR1223, HR1316, HR1330 and HR1332 were derived from these strains by

The GenBank accession numbers for the sequences of the colicin G and colicin H determinants are AJ515251 and AJ515252, respectively.

Table 1. Strains, plasmids and phage

Strain/plasmid/phage	Relevant marker	Reference
Strains		
<i>E. coli</i> DSM 6601	Microcin M and H47 determinant	Mutaflor®/Ardeypharm, Germany
SK22D	As DSM 6601, but $\Delta mchDEF$	This work
H5445	As DSM 6601, but <i>mchF</i> ::Mud1	This work
<i>E. coli</i> CA46	Colicin G	Davies & Reeves (1975)
<i>E. coli</i> CA58	Colicin H	Davies & Reeves (1975)
EN777	pColV-K311 <i>zz1</i> ::Tn10	Gross <i>et al.</i> (1984)
<i>E. coli</i> K-12		
H1443	As MC4100, but <i>aroB</i>	Hantke (1990)
H1673	As W3110, but <i>fur-31 zbf</i> ::Tn10	Stojiljkovic <i>et al.</i> (1994)
H1717	As H1443, but <i>fhuF</i> :: λ placMu53	Stojiljkovic <i>et al.</i> (1994)
H1728	As H1443, but <i>fiu</i> ::MudX <i>cir</i>	Hantke (1990)
H1875	As H1443, but <i>cir</i> ::MudX <i>fepA</i> ::Tn10	Hantke (1990)
H1876	As H1443, but <i>cir fepA</i> ::Tn10 <i>fiu</i> ::MudX	Hantke (1990)
H1877	As H1443, but <i>fepA</i> ::Tn10 <i>fiu</i> ::MudX	Hantke (1990)
H5316	As MC4100 but <i>entC</i> ::MudX <i>fur-31 zbd</i> ::Tn10	This work
H5317	As MC4100, but <i>entC</i> ::MudX <i>tonB trp</i> ::Tn10	This work
MC4100	Δlac	Casadaban & Cohen (1979)
SIP401	As MC4100, but <i>fur-28</i>	Patzer & Hantke (2001)
SIP880	As H1443, but <i>fepA</i> ::MudX	This work
RYC1000	As MC4100, but $\Delta rbs-7 recA56 gyrA$	Genilloud <i>et al.</i> (1984)
SIP1003	λ' lacZYA fusion of bp 640–877	This work
SIP1004	λ' lacZYA fusion of bp 77–877	This work
SIP1005	λ' lacZYA fusion of bp 877–77	This work
SIP1010	λ' lacZYA fusion of bp 877–640	This work
SIP1011	λ' lacZYA fusion of bp 877–640 <i>fur-28</i>	This work
SIP1013	λ' lacZYA fusion of bp 640–877 <i>fur-28</i>	This work
SIP1014	λ' lacZYA fusion of bp 77–877 <i>fur-28</i>	This work
SIP1015	λ' lacZYA fusion of bp 877–77 <i>fur-28</i>	This work
WL311	Δlac	P. Howard, Dept Microbiol., Univ. Saskatchewan
<i>Klebsiella pneumoniae</i> E492	Microcin E492	Lagos <i>et al.</i> (1999)
<i>Salmonella enterica</i> serovar Typhimurium LT2		
TA2700	<i>ent fhuC</i>	Rabsch <i>et al.</i> (1999)
H2700	<i>ent</i>	This work
WR1223	<i>ent fhuC iroN</i> ::pGP704	Rabsch <i>et al.</i> (1999)
HR1223	<i>ent iroN</i> ::pGP704	This work
WR1316	<i>ent fhuC fepA</i> ::Tn10	Rabsch <i>et al.</i> (1999)
HR1316	<i>ent fepA</i> ::Tn10	This work
WR1330	<i>ent fhuC fepA</i> ::Tn10 <i>iroN</i> ::pGP704 <i>cir</i> :: <i>kan</i>	Rabsch <i>et al.</i> (1999)
HR1330	<i>ent fepA</i> ::Tn10 <i>iroN</i> ::pGP704 <i>cir</i> :: <i>kan</i>	This work
WR1332	<i>ent fhuC iroN</i> ::pGP704 <i>fepA</i> ::Tn10	Rabsch <i>et al.</i> (1999)
HR1332	<i>ent iroN</i> ::pGP704 <i>fepA</i> ::Tn10	This work
<i>S. enterica</i> serovar Stanleyville 207/81		
WR1359	<i>iroN</i> ::pGP704	Hantke <i>et al.</i> (2003)
WR1361	<i>cir</i> ::MudJ	Hantke <i>et al.</i> (2003)
WR1363	<i>iroN</i> ::pGP704 <i>cir</i> ::MudJ	Hantke <i>et al.</i> (2003)
WR1366	<i>iroN</i> ::pGP704 <i>fepA</i> ::Tn10	Hantke <i>et al.</i> (2003)
WR1367	<i>cir</i> ::MudJ <i>fepA</i> ::Tn10	Hantke <i>et al.</i> (2003)
WR1368	<i>iroN</i> ::pGP704 <i>cir</i> ::MudJ <i>fepA</i> ::Tn10	Hantke <i>et al.</i> (2003)
Plasmids		
pEX4	Microcin H47 [Amp ^R]	Lavina <i>et al.</i> (1990)
pGOB18	Microcin 24 [Amp ^R]	T. S. Frana, Coll. Vet. Med., Iowa State Univ.

Table 1. cont.

Strain/plasmid/phage	Relevant marker	Reference
pHM1	Microcins M and H47 [Cam ^R]	M. R. Baquero, D. Bravo & F. Moreno, unpublished
pSP61/18	pHSG575 <i>fur</i>	Patzer & Hantke (2001)
pSP110/63, pSP110/73	DSM 6601 microcin operon region bp 5945–8939 cloned into pUC19, both orientations	This work
pSP110/174, pSP110/196	DSM 6601 microcin operon region bp 2149–5744 cloned into pUC19, both orientations	This work
pSP110/270, pSP110/271	DSM 6601 microcin operon region bp 198–2320 cloned into pUC19, both orientations	This work
pSP112/1, pSP112/4	DSM 6601 microcin operon region bp 7083–7592 cloned into pUC19	This work
pSP112/9, pSP112/12	DSM 6601 microcin operon region bp 7425–7592 cloned into pUC19	This work
pSP112/14, pSP112/19	DSM 6601 microcin operon region bp 7926–9673 cloned into pUC19	This work
pSP112/15, pSP112/17	DSM 6601 microcin operon region bp 7926–9673 cloned into pUC19, other orientation	This work
pSP112/134	CA46 <i>mchXIBCDEF mcmIC</i> cloned into pSU19	This work
pSP112/149	DSM 6601 <i>mchXIBCDEF mcmIC</i> cloned into pSU19	This work
pSP112/199	DSM 6601 <i>mchDEF mcmI mcmA-S92C</i> on pSU19	This work
pSP119/58, pSP119/60	DSM 6601 microcin operon region bp 640–877 cloned into pUC19	This work
pSP119/35, pSP119/38	DSM 6601 microcin operon region bp 822–844 cloned into pUC18, both orientations	This work
pSP119/52, pSP119/53	DSM 6601 microcin operon region bp 822–844 cloned into pUC19, both orientations	This work
pRS415	<i>lacZYA</i> operon fusion vector	Simons <i>et al.</i> (1987)
pUC19, pUC18		Laboratory stock
Phage		
λRS45		Simons <i>et al.</i> (1987)

transduction to *fluC*⁺ with a P22 lysate from *S. enterica* serovar Typhimurium LT2. *E. coli* strain SK22D was obtained by selection at 42 °C for deletions of the temperature-sensitive Mud1 inserted in *E. coli* H5445. The deletion was localized by PCR with various primers and found to begin between bp 2320 and 2640 and end between bp 5675 and 5720. Thus *mchC*, *mchD*, *mchE* and *mchF* are missing.

For construction of the chromosomal *lacZ* fusions, a PCR product of the region to be studied was inserted in both directions into the *Sma*I site of plasmid pRS415, recombined into the phage λRS45 in front of the *lacZYA* genes, and introduced into *E. coli* MC4100 by infection with the recombinant λ phage and selection of lysogens. The *E. coli fur* mutants SIP1011, SIP1013, SIP1014 and SIP1015 carry the *fur-28* allele (Patzer & Hantke, 2001), associated with *zbf-15::Tn10* as a marker for P1 transduction; the *fur-28* allele has a 70 bp deletion, which yields an inactive Fur protein.

To obtain the plasmids pSP112/134 and pSP112/149, the PCR products obtained with primers *cva38* and *cva13* and chromosomal DNA of strains CA46 and DSM 6601, respectively, were each cloned into the *Sma*I site of pSU19.

Plasmid pEX4 (Amp^r), encoding microcin H47, was kindly supplied by M. Lavina (Rodriguez *et al.*, 1999). pHM1 was constructed by ligating a 5.8 kb *Eco*RI/*Bam*HI fragment containing the genes

mcmL, *mcmK*, *mchX* and part of *mcmI* from pEX4 into a plasmid encoding the rest of *mchI* and the rest of the microcin M determinant from strain DSM 6601 (Fig. 1) in a pACYC184 vector (details will be described elsewhere).

Bacteria were grown in TY medium (8 g tryptone l⁻¹, 5 g yeast extract l⁻¹ and 5 g NaCl l⁻¹) or in M63 glucose minimal medium (Miller, 1972). Microcin production was tested on TY plates containing 50 μM desferal or on nutrient broth dipyriddy plates (8 g nutrient broth l⁻¹, 5 g NaCl l⁻¹, 15 g Difco agar l⁻¹, and 0.2 mM 2,2'-dipyridyl). After overnight growth of the microcin-producing strain on these plates, the appropriate indicator strain was overlaid in 2.5 ml TY soft agar, and zones of growth inhibition were measured after 16 h.

Recombinant DNA techniques. Standard procedures (Ausubel *et al.*, 1996) or those recommended by commercial suppliers were followed for isolation of chromosomal and plasmid DNA, cleavage with restriction endonucleases, DNA modification, ligation, transformation and agarose gel electrophoresis. DNA was sequenced by the dideoxy chain-termination method using the AutoRead sequencing kit or the Thermo Sequenase Cy5 dye terminator kit for cycle sequencing and the ALF sequencer (Pharmacia Biotech). A Robo Cycler Gradient 96 (Stratagene) was used for PCR. Oligonucleotides were synthesized by Eurogentec (Seraing, Belgium).

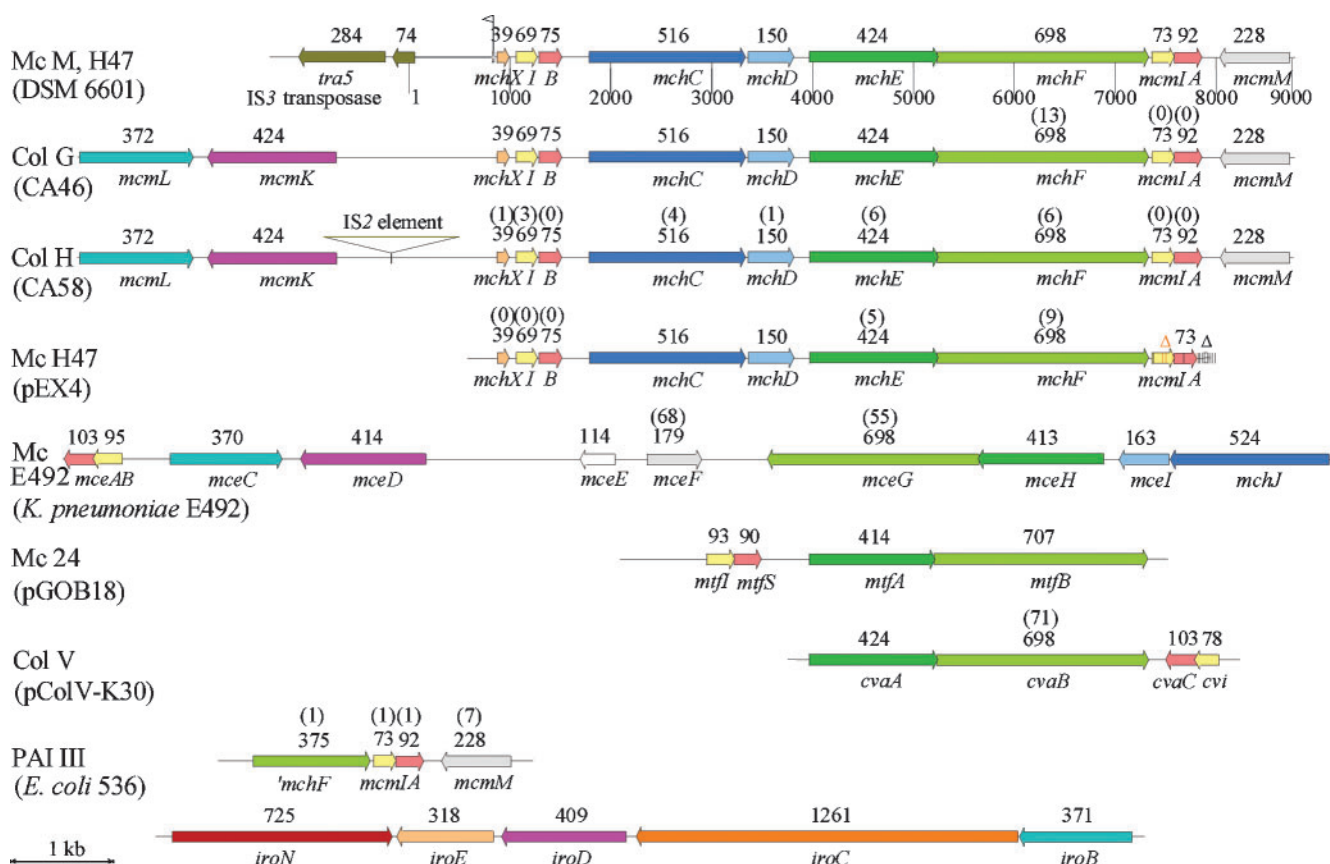


Fig. 1. Comparison of microcin gene clusters containing *cvaA*- and *cvaB*-like genes: Mc M, H47, microcins M and H47 from *E. coli* DSM 6601; Col G, colicin G from *E. coli* CA46; Col H, colicin H from *E. coli* CA58; Mc H47, microcin H47 from pEX4 (Azpiroz *et al.*, 2001; Rodriguez *et al.*, 1999); Mc E492, microcin E492 from *Klebsiella pneumoniae* E492 (Lagos *et al.*, 2001); Mc 24, microcin 24 from *E. coli* 2424 (GenBank accession number U47048); Col V, colicin V from pColV-K30 (Gilson *et al.*, 1990); PAI III, pathogenicity island III (536) of the uropathogenic *E. coli* 536 (Dobrindt *et al.*, 2001). In all systems, the genes for the export of microcins *cvaA* and *cvaB* (or their homologues) are coupled. Orientation and location of the structural genes for the microcins and their immunity proteins differ. The numbers above the genes denote the size of the encoded protein in amino acid residues; the number of mutated amino acids compared to the proteins of DSM 6601 (first line) is given in parentheses. The numbers below the Mc M, H47 line indicate the bp numbers of the nucleotide sequence. The Fur box in front of *mchX* is marked by a small flag.

The sequences of the colicin G and colicin H determinants have been deposited under the accession numbers AJ515251 and AJ515252, respectively, in the EMBL/GenBank database. The numbering of the bp corresponds to the numbering in Fig. 1.

RESULTS

Identification of the microcin-encoding gene cluster

Microcin production by strain DSM 6601 was observed on M63 minimal medium agar plates and on nutrient broth/dipyridyl agar plates by overlaying colonies of strain DSM 6601 with microcin-sensitive strains, such as *E. coli* H5316. Zones of growth inhibition of about 4 mm formed. The growth-inhibiting activity was sensitive to chloroform vapour as has been described (Papavassiliou,

1961). Owing to low activity and instability, purification of the activity from liquid cultures was difficult. For further characterization of the toxin, we screened for mutants of strain DSM 6601 that were unable to produce this activity. To identify the encoding genes, strain DSM 6601 was mutagenized with the transposing phage Mud1 (Amp, *lac*) (Casadaban & Cohen, 1979). One out of 50 mutants, strain H5445, did not produce the bactericidal activity.

DNA with the insertion site of Mud1 was amplified by PCR and cloned using a primer complementary to the right end of Mud1 (Patzer & Hantke, 1998). Sequencing revealed high similarity to *cvaA* (96%) and *cvaB* (90%) (Table 2), which encode two proteins that export colicin V (Gilson *et al.*, 1990). Colicin V is in fact a microcin (Baquero *et al.*, 1978), but the designation colicin V will be used as mostly this name is found in the literature. Two other recently

Table 2. Comparison of CvaA and CvaB to microcin export proteins

Percentages of identical amino acids are given. Export of colicin V is mediated by CvaA/B, of microcins H47 and M by MchE/F, of microcin E 492 by MceH/G, and of microcin 24 by MtfA/B.

	Membrane fusion proteins				ABC exporter				
	CvaA	MchE	MceH	MtfA	CvaB	MchF	MceG	MtfB	
CvaA	100	97	91	71	CvaB	100	89	87	73
MchE		100	94	71	MchF		100	92	75
MceH			100	72	MceG		100	77	
MtfA				100	MtfB			100	

sequenced genes, *mchE* and *mchF*, encoding the exporter for microcin H47 (Azpiroz *et al.*, 2001), are 98 and 99% identical to the *cvaA* and *cvaB* genes, respectively, from strain DSM 6601. We therefore designate the genes *mchE* and *mchF*.

Downstream of *mchF*, the genes encoding microcin M, *mcmA* [the name was changed from *mcmC* (Braun *et al.*, 2002) to *mcmA* to follow the nomenclature of microcin genes], and the immunity protein, *mcmI*, were found; the proteins are distantly related to the colicin V activity and immunity proteins (Table 3, Fig. 1). Further downstream of *mcmI* and *mcmA*, with opposite transcription polarity, the *mcmM* gene was found, which encodes a protein with 62% identity to MceF, a protein involved in maturation of microcin E492 (Lagos *et al.*, 2001). McmM also has some similarity (37% identity) to the protein CvpA, which is encoded on the *E. coli* K-12 chromosome and is required in an unknown way for colicin V production from plasmid pColV-K30 (Fath *et al.*, 1989). The microcin determinant ends upstream of *mcmM*, beginning at bp 10362; the sequence following the microcin determinant is nearly identical to that of the *fim* operon in *E. coli* 4787 O155:v165:f165 starting at bp 43 (GenBank accession number U098571).

The similarity to the *cva* operon ceased in front of *mchE*, upstream of bp 3968. Two open reading frames similar in size to *mchD* and *mchC* (Fig. 1) of the microcin H47 gene

cluster (Rodriguez & Lavina, 1998) were detected. The protein encoded by *mchD* is 150 amino acids long and has weak sequence similarities to acyltransferases, such as HlyC, encoded in the *E. coli* haemolysin operon, necessary for acylation of haemolysin (Stanley *et al.*, 1998). In a BLAST search, no proteins with known function were found to be similar to MchC.

Sequence analysis of the region upstream of *mchC* revealed nearly 100% identity to *mchB*, the gene encoding microcin H47; to *mchI*, the gene encoding the microcin H47 immunity protein; and to *mchX*, a gene with unknown function (Rodriguez *et al.*, 1999; Rodriguez & Lavina, 1998). The similarity to the *mch* operon ceased 756 bp upstream of *mchX*. An IS3 element with high sequence similarity to the pathogenicity island SHI-2 of *Shigella flexneri* was found (GenBank accession number AF141323.1, bp 3867–5111) (Moss *et al.*, 1999). This pathogenicity island is near the *selC* tRNA locus and encodes the aerobactin iron uptake system and ColV immunity in *Shigella flexneri*.

The entire DNA segment sequenced is similar to a part of the recently published genome sequence of the uropathogenic *E. coli* CFT073 (Welch *et al.*, 2002), in which, however, genes *mcmA* and *mcmI* were not annotated (GenBank accession number AE016758, bp 273878–284628).

These results indicated that strain DSM 6601 probably synthesizes microcins H47 and M, which are then exported

Table 3. Comparison of different microcins and their immunity proteins

Percentages of identical amino acids are given. McmA is microcin M, and McmI is the immunity protein; MchB is microcin H47, and MchI is the immunity protein; CvaC is colicin V, and Cvi is the immunity protein; MceA is microcin E492, and MceB is the immunity protein; MtfS is microcin 24, and MtfI is the immunity protein.

	Microcins					Immunity proteins				
	McmA	MchB	CvaC	MceA	MtfS	McmI	MchI	Cvi	MceB	MtfI
McmA	100	38	35	28	<20	McmI	100	16	22	22
MchB		100	22	40	27	MchI		100	<20	35
CvaC			100	<20	<20	Cvi		100	<20	<20
MceA				100	45	MceB			100	39
MtfS					100	MtfI				100

by a common export machinery also found in colicin-V-producing strains. In these strains, the ATPase domain of the cytoplasmic membrane protein CvaB energizes microcin export. In addition, CvaB contributes to cleavage of the microcins after a glycine-glycine (or alanine-glycine) motif during export (Havarstein *et al.*, 1995). CvaA is anchored in the cytoplasmic membrane and mainly located in the periplasm, where it serves as a connector or membrane fusion protein to the outer-membrane protein TolC. The ABC transporter CvaB is connected by CvaA to the trimeric outer-membrane protein TolC, which forms a single β -barrel in the outer membrane with a bundle of α -helices extending into the periplasm (Koronakis *et al.*, 2000). The CvaA/CvaB/TolC channel allows the secretion of microcins across two membranes, which is a general feature of these RND-type exporters (Tseng *et al.*, 1999).

Partial sequencing of plasmid pEX4 (Azpiroz *et al.*, 2001), which encodes microcin H47, indicated that the high similarity observed for the sequence encoding MchXIBCDEF extended beyond the region deposited in the database. A comparison of the *mcmI* and *mcmA* genes on pEX4 to the sequence of strain DSM 6601 revealed several mutations in pEX4 that do not allow the expression of an active microcin M and its immunity protein.

Colicins G and H determinants encode the same microcins

Colicins G and H had the same receptor specificities as microcins H47 and M (see Table 6 and below). The fragments of the microcin M/H47 gene cluster from the

colicin-G- and colicin H-producing strains were amplified by PCR and the gene clusters were cloned. Sequencing of the entire determinants revealed that the genetic organization and the sequences were very similar to those of the microcin gene clusters of strain DSM 6601 and plasmid pEX4 (Fig. 1).

However, in the colicin G and the colicin H gene clusters, upstream of *mchX*, two additional genes were identified, *mcmK* and *mcmL* (Fig. 1), which showed similarities to *mceC* and *mceD*, respectively, of the microcin E492 determinant and to genes from the *iroA* cluster (Table 4). The *iroA* gene cluster, originally found in *S. enterica* serovar Typhimurium (Bäumler *et al.*, 1996), is also found in certain uropathogenic *E. coli* strains (Dobrindt *et al.*, 2001; Welch *et al.*, 2002) and encodes part of the catecholate siderophore salmochelin synthesis and uptake system (Hantke *et al.*, 2003). Especially the similarities of McmL and McmK to the *E. coli* IroB (99%) and IroD (55%) proteins, respectively, are striking (Table 4). IroB has similarities to glycosyltransferases involved in antibiotic biosynthesis in *Streptomyces*, while IroD and McmK have weak similarities to Fes, the enterochelin esterase. The genes *mcmK* and *mcmL* were not found upstream of *mchX* in strains DSM 6601 and CFT073, but in both strains an *iroA* gene cluster is present, as demonstrated by the production of salmochelin (K. Hantke & G. Winkelmann, unpublished). It is possible that in these cases IroB and IroD fulfil the functions of McmK and McmL.

An IS2 element was found between *mchX* and *mcmK* in the colicin H gene cluster. This is reminiscent of the IS3-like sequences found in strains DSM 6601 and CFT073 at a

Table 4. Comparison of *E. coli* Iro proteins with *Salmonella* Iro proteins and proteins involved in microcin biosynthesis

In addition, the similarity to some better-defined homologues is provided. DHBS is 2,3-dihydroxybenzoylserine.

<i>iro</i> gene from <i>E. coli</i> 536	Homologues	Size (aa)	Putative function of the proteins	Identity (%)
<i>iroB</i>		371	Transglycosylase	100
	<i>iroB</i> <i>Salmonella</i>	371	Transglycosylase	72
	<i>mceC</i> <i>Klebsiella</i>	370	Maturation of microcin E492	84
	<i>mcmL</i> <i>E. coli</i>	372	Maturation of microcins M and H	99
<i>iroC</i>		1261	ABC exporter	100
	<i>iroC</i> <i>Salmonella</i>	1218	ABC exporter	79
	<i>MDR1</i> <i>Homo sapiens</i>	1280	ABC exporter, multidrug resistance protein 1	28
<i>iroD</i>		409	Similar to enterochelin esterase	100
	<i>iroD</i> <i>Salmonella</i>	414	Similar to enterochelin esterase	68
	<i>mceD</i> <i>Klebsiella</i>	414	Maturation of microcin E492	56
	<i>mcmK</i> <i>E. coli</i>	424	Maturation of microcins M and H	55
	<i>fes</i> <i>E. coli</i>	374	Enterochelin esterase	27
<i>iroE</i>		318	Periplasmic hydrolase	100
	<i>iroE</i> <i>Salmonella</i>	311	Periplasmic hydrolase	61
<i>iroN</i>		725	Catecholate siderophore receptor	100
	<i>iroN</i> <i>Salmonella</i>	726	Catecholate siderophore receptor	82
	<i>fepA</i> <i>E. coli</i>	746	Enterochelin receptor	50
	<i>cir</i> <i>E. coli</i>	663	DHBS-catecholate siderophore receptor	32
	<i>fiu</i> <i>E. coli</i>	760	DHBS-catecholate siderophore receptor	22

Table 5. Immunity analysis

The microcin-producing strains were grown on TY plates containing 50 μ M desferal for 36 h. A 0.05 ml aliquot of the appropriate overnight-grown indicator strain was overlaid in 3 ml soft water agar, and the zone of growth inhibition was measured after 16 h.

Indicator strain	Inhibition zone (mm) with microcin-producing strain/plasmid:				
	DSM 6601 (microcin H47, M)	CA46 (colicin G); CA58 (colicin H)	MC4100 pSP112/199 (microcin M)	RYC1000 pEX4 (microcin H47)	MC4100 pSP112/134; pSP112/149 (microcin H47, M)
SIP401 <i>fur</i>	3	14	8	15	2
MC4100; RYC1000	2	12	7	14	1
DSM 6601 (microcin H47, M)	0	0	0	0	0
CA46 (colicin G); CA58 (colicin H)	0	0	0	0	0
MC4100 pSP112/134; pSP112/149 (microcin H47, M)	0	0	0	0	0
MC4100 pSP112/199 (microcin M)	1	7	0	14	1
RYC1000 pEX4 (microcin H47)	1	5	7	0	1

similar location. IS elements are known to stimulate recombination, deletion, inversion and transposition.

Cloning of the microcin gene cluster

It was difficult to clone the microcin M operon in *E. coli* K-12 in an active form. For sequencing, it was necessary to clone DNA fragments carrying parts of the microcin region repeatedly since the DNA often contained mutations. Only a few larger clones showed microbicidal activity when the microcin M operon was associated with the microcin H47 operon (pSP112/134, pSP112/149; Table 5). The inhibitory activity of pSP112/134 and pSP112/149 and of strain DSM 6601 was weak, but the inhibition zone was more visible with a *fur* mutant as indicator strain (SIP401, Table 5), in which the receptors for microcin uptake were overexpressed.

However, from hundreds of clones tested, only plasmid pSP112/199, which carried genes *mchDEF mcmIA* (Fig. 1), showed a stable microcin production, which indicates that microcin M alone has microbicidal activity. Sequencing revealed that a mutation had occurred that changed the last amino acid of McmA from Ser to Cys. This mutation allowed stable expression of the microcin McmA-S92C with high bactericidal activity.

Microcins H47 and M are the bactericidal determinants of colicin G and colicin H activity

The immunity proteins MchI (microcin H47) and McmI (microcin M) are specific for their microcin activity proteins MchB (microcin H47) and McmA (microcin M), respectively. *E. coli* MC4100(pSP112/199), which produced the immunity protein only for microcin M, was completely insensitive to the activity of microcin M (S92C) produced from pSP112/199, but was sensitive to microcin H47 activity secreted by *E. coli* RYC1000(pEX4) (Table 5). In contrast, the strain carrying pEX4, which produced the immunity protein only of the microcin H47 cluster, was protected

against the microcin H47 encoded on pEX4, but was not protected against the microcin M (S92C) secreted by *E. coli* MC4100(pSP112/199) (Table 5).

E. coli MC4100(pSP112/134) and *E. coli* MC4100(pSP112/149), both of which produced both microcin H47 and M immunity proteins, were insensitive to the colicin G producer *E. coli* CA46 and the colicin H producer *E. coli* CA58, which showed that the bactericidal activity produced by strains CA46 and CA58 can be completely overcome by the microcin H47 and M immunity proteins (Table 5). Thus, microcins H47 and M are the only bacteriocin determinants of strains CA46 and CA58. Microcin H47 and microcin M were both produced by strains CA46 and CA58, as shown by the zone of growth inhibition when these strains were overlaid with an indicator strain producing the microcin M immunity protein [*E. coli* MC4100(pSP112/199)] or the microcin H47 immunity protein [*E. coli* RYC1000(pEX4)] (Table 5).

Receptor specificity of microcins H47, M, E492 and 24, and colicins G and H

To elucidate the receptor(s) necessary for the uptake of the microcins, *E. coli* strains DSM 6601, RYC1000(pEX4), CA46, CA58, E492, EN777(pColV), and microcin 24 from *E. coli* H5513(pGOB18), were tested against different strains with defined receptor mutations. In addition, plasmid pHM1, which carried *mcmL mcmK mchX* from pEX4 and *mchBCDEF mcmIA* from strain DSM 6601 (M. R. Baquero, D. Bravo & F. Moreno, unpublished), was included. To test the receptor specificities of the different microcins, a set of siderophore receptor mutants was used. The zone of growth inhibition was determined with an agar overlay technique.

The microcins did not kill *tonB* mutants (Table 6), as has been described for colicins G, H, V (Pugsley & Reeves, 1976) and E492 (Pugsley *et al.*, 1986). Most TonB-dependent colicins and microcins use siderophore receptors to cross the outer membrane. Microcin 24 is the only one of the

Table 6. Inhibition of growth by microcins

Microcin-producing strains were grown in TY medium containing 50 µM desferal to create iron-poor conditions; microcin-producing strains were killed by a 30 min exposure to chloroform vapour, which did not influence the observed activities. A 0.05 ml aliquot of the appropriate overnight-grown indicator strain was overlaid in 2.5 ml TY soft agar, and the zone of growth inhibition was measured in mm the next day. *E. coli* RYC1000 was used as host for plasmids pHM1 and pEX4; *E. coli* MC4100 was used as host for the microcin-M-S92C-encoding plasmid pSP112/199. NT, Not tested.

Indicator strain	Inhibition zone (mm) with microcin-producing strain/plasmid:							
	pHM1	pEX4	DSM 6601	CA46 ColG	pSP112/199	E492	EN777 ColV	pGOB18 Mc 24
<i>E. coli</i> K-12								
W3110	12	12	4	10	5	NT	NT	10
SIP880 <i>fepA</i>	14	2	1	7	6	5	12	NT
H1728 <i>cir fiu</i>	16	4	1	9	5	9	0	NT
H1875 <i>fepA cir</i>	14	2	1	7	6	7	0	NT
H1877 <i>fepA fiu</i>	12	2	1	5	1	4	12	NT
H1876 <i>fepA cir fiu</i>	0	0	0	0	0	0	0	NT
H1673 <i>fur</i>	13	10	3	10	6	8	10	nt
H5317 <i>tonB</i>	0	0	0	0	0	0	0	0
<i>S. enterica</i> serovar Stanleyville								
207/81	10	10	0	2	0	10	5	10
WR1359 <i>iroN</i>	4	4	0	2	0	3	5	11
WR1361 <i>cir</i>	10	10	1	0	0	8	0	10
WR1366 <i>fepA iroN</i>	6	4	0	2	0	3	5	10
WR1367 <i>fepA cir</i>	11	10	1	2	0	9	0	10
WR1368 <i>fepA cir iroN</i>	0	0	0	0	0	0	0	10
<i>S. enterica</i> serovar Typhimurium LT2								
H2700	10	9	0	2	NT	5	7	11
HR1223 <i>iroN</i>	9	6	0	4	NT	NT	7	11
HR1316 <i>fepA</i>	8	5	0	3	NT	NT	8	12
HR1332 <i>fepA iroN</i>	7	5	0	3	NT	NT	9	12
HR1330 <i>fepA iroN cir</i>	0	0	0	0	NT	NT	0	12

above-mentioned microcins that is dependent on FhuA, the receptor for the hydroxamate siderophore ferrichrome. Microcin J25 is also taken up via FhuA in *E. coli* (Killmann *et al.*, 2001; Salomon & Farias, 1993). *S. enterica* serovar Typhimurium, however, is microcin-J25-resistant and microcin-24-sensitive. *S. enterica* serovar Typhimurium *fhuA* or *tonB* mutants are microcin-24-resistant, which shows that microcin 24 also recognizes FhuA from *S. enterica* serovar Typhimurium LT2 and has a broader host specificity than microcin J25. Microcin 24 was used as a control during tests of the other microcins.

Previously, it has been observed that Fiu receptor mutants are less sensitive to colicins G and H than the parent strains (Hantke, 1983). The Fiu protein belongs to the large family of TonB-dependent outer-membrane receptors that transport siderophores across the outer membrane of Gram-negative bacteria (Braun *et al.*, 1998). The natural siderophore substrates of Fiu are catecholate siderophores (Hantke, 1990; Curtis *et al.*, 1988). The receptor seems to accept very diverse substrates since catechol-cephalosporins are also accumulated by Fiu in a TonB-dependent manner

(Curtis *et al.*, 1988). However, *fiu* mutants were only partially resistant to colicins G and H. In *E. coli*, there is a second TonB-dependent receptor, Cir, which has nearly the same substrate specificity as Fiu (Hantke, 1990). In addition, Cir is the receptor of colicins V and I. The third catecholate-specific receptor is FepA, which recognizes iron-enterochelin (enterobactin) (Earhart, 1996), the iron complex of the general *E. coli* siderophore, which is produced under low-iron growth conditions. In addition, FepA serves as receptor for the TonB-dependent colicins B and D.

On iron-rich tryptone/yeast extract medium (TY), strains DSM 6601 and EN777(pColV) produced no or very low amounts of microcin, respectively. On TY medium containing 50 µM of the iron-scavenging desferal (desferrioxamine B mesylate), high activities of colicin V were observed. This is in accordance with expectation, since colicin V production is derepressed under low-iron growth conditions (Chehade & Braun, 1988), such as when Fe³⁺ is complexed with the ferrioxamine B of desferal. Ferrioxamine B is a poor iron source for *E. coli* K-12 (Patzer & Hantke, 1999), which lacks a receptor for this

siderophore in its outer membrane (Bäumler & Hantke, 1992), and uptake via the ABC transporter FhuBCD is not efficient owing to the low affinity of FhuD for ferrioxamine B.

The receptor specificities for microcins H47 and M were not always as clear-cut as for colicin V. Microcin H47 produced by *E. coli* RYC1000(pEX4) seemed to use mainly FepA as receptor. The *cir fiu* double mutant was more sensitive to microcin H47 than the *fepA fiu* or the *fepA cir* mutants. Only the triple mutant *fepA cir fiu* was completely resistant, which indicated that all three receptors can be used by microcin H47 to gain access with different efficiencies to the cell. Strain DSM 6601 produced small amounts of microcins only under iron-poor conditions. Again, only the triple mutant *fepA cir fiu* and the *tonB* mutant were resistant to both microcins (Table 6). *E. coli* RYC1000(pHM1) produced much more microcins H47 and M than strains DSM 6601 and RYC1000(pEX4). Only the triple mutant was resistant to both microcins.

The microcin-M-S92C-producing plasmid pSP112/199 was transformed into *E. coli* MC4100 and into *E. coli* RYC1000, and the activity was compared to that of a strain carrying pHM1. For microcin M-S92C, the receptor proteins FepA and Fiu were most important (Table 6); strain H1877, containing only the Cir receptor protein in its outer membrane, showed only residual sensitivity to microcin M-S92C. On strains of *S. enterica* serovar Stanleyville, no activity was observed. Either microcin M is not active against *S. enterica* serovar Stanleyville or the amounts of microcin produced were too low.

In contrast, mutant SK22D $\Delta mchDEF$ derived from strain DSM 6601 was still sensitive to the high amounts of microcins produced by strain RYC1000(pHM1), although the reduction in size of the zone of growth inhibition from 12 to 6 mm reflected a partial immunity. This could be explained by a polar effect of the deletion on the expression of the downstream immunity gene *mcmI*.

Microcins of strain CA46 (colicin G producer) and of strain CA58 (colicin H producer) should be the same, and indeed, the sensitivity patterns were nearly identical; therefore, only the data for strain CA46 are shown in Table 6. The amount of microcins produced by strain CA46 was lower than that of *E. coli* RYC1000(pHM1). Again, the triple mutant *fepA cir fiu* was completely resistant to the microcins from these three strains (Table 6).

Iron is a microcin receptor in *Salmonella*

From the genome sequence of *Salmonella* strains, it is known that these strains have FepA and Cir proteins highly similar to those of *E. coli* (81 and 88% identity, respectively). Various strains of *S. enterica* serovar Typhimurium LT2 were tested for their sensitivity to microcins (Table 6). No growth inhibition zones were observed when colonies of *E. coli* DSM 6601 were overlaid with *S. enterica* serovar

Typhimurium LT2. Strains producing colicin G and H showed growth inhibition zones smaller than those produced on *E. coli* H1673 *fur*. Since it is known that *Salmonella* strains synthesize IroN as the third catecholate-specific receptor (Rabsch *et al.*, 1999), mutants of this type were included in the test. The FepA mutant showed a reduced sensitivity, similar to that of the double mutant *iroN fepA*; however, only the triple mutant *cir fepA iroN* was completely insensitive to the microcins. A slightly different picture was observed with strains derived from *S. enterica* serovar Stanleyville, where IroN is clearly the main receptor for these microcins, followed by Cir and then FepA. The slightly different reaction of the two *Salmonella* species might be explained by sequence differences in the receptor proteins or in the regulation of receptor expression and activity.

In conclusion, strain DSM 6601 produced very low amounts of microcins compared to other microcin-H47- and microcin-M-producing strains, most likely owing to the loss of the *mcmL* and *mcmK* genes. Using different primers from the *mcmL/mcmK* region, no PCR fragments were obtained from strain DSM 6601 chromosomal DNA, which indicates that these genes are not present; however, very similar genes, *iroB* (99% identity to *mcmL*) and *iroD* (55% identity to *mcmK*), are found in the *iro* gene cluster, which might complement the loss of *mcmL* and *mcmK*. It is possible that the strain has lost this DNA region during subculturing since 1917. It is interesting to note that part of an IS3 element is found in strains DSM 6055 and CFT073 upstream of *mchX*, and an IS2 element is found in the colicin H determinant between *mchX* and *mcmK*.

Iron regulation

The production of colicin V is derepressed under low-iron growth conditions (Chehade & Braun, 1988; Boyer & Tai, 1998). Similarly, it was observed here that optimal microcin production was obtained with strain DSM 6601 only on low-iron media (M63 minimal medium, nutrient broth containing dipyriddy, or TY containing 50 μ M desferal). For this reason, the nucleotide sequence of the microcin-encoding region was searched for binding sites of Fur, the global iron regulator. Fur binding sites have been found in the promoter region of several iron-regulated genes (Stojiljkovic *et al.*, 1994; De Lorenzo *et al.*, 1987), and a consensus sequence, GATAATGATAATCATTATC, has been derived. A computer search revealed possible Fur boxes at bp 824–842 in the promoter region of *mchX*, at bp 7247–7265 in the promoter region of *mcmI*, at bp 9386–9404 in the promoter region of *mcmM*, at bp 4841–4859 and at bp 4847–4865.

To test for Fur binding, the Fur titration assay (Stojiljkovic *et al.*, 1994) was used, in which DNA with a potential Fur box is cloned in a high-copy-number plasmid and introduced into a strain with an iron- and Fur-regulated *lacZ* operon fusion. The chromosomally encoded Fur protein binds to the Fur box on the plasmid, and the

iron-regulated *fhuF-lacZ* fusion is derepressed; this can be visualized on MacConkey agar plates by the formation of red colonies. Strains transformed with the vector alone remain white on these plates since there is enough Fur to repress the *fhuF-lacZ* fusion.

The indicator strain *E. coli* H1717 (*fhuF-lacZ*) transformed with the region bp 5945–8939 cloned into pUC19, yielding pSP110/63 and pSP110/73 (fragment in both orientations), was weakly active in the assay. Subcloning revealed that regions bp 7083–7592 (pSP112/1, pSP112/4), bp 7425–7592 (pSP112/9 and pSP112/12, fragment in both orientations), and bp 2149–5744 (pSP110/174 and pSP110/196) were not active *in vivo*, whereas region bp 7926–9673 was weakly active in one orientation (pSP112/14, pSP112/19) and inactive in the other orientation (pSP112/15 and pSP112/17). However, region bp –198–2320 (pSP110/270 and pSP110/271) showed high activity. The active region was narrowed down to bp 640–877 (pSP119/58 and pSP119/60 in the pUC19 vector), which was as active as the entire region. The region bp 822–844 showed only weak derepression (pSP119/38 and pSP119/35, fragment in both orientations in pUC18; and pSP119/52 and pSP119/53, fragment in both orientations in pUC19), which indicated that it was too small to obtain full iron regulation.

Only fragments containing the promoter region of *mchX* (bp 640–877) showed high activity in the Fur titration assay. All the other Fur binding sites had low or no activity in the *in vivo* titration assay. To verify these results, the potential promoters were studied in single copy as a chromosomal *lacZYA* fusion. The influence of iron on the expression of the reporter gene *lacZ* was tested on MacConkey/lactose agar plates. Chromosomal fusions were preferred since undesired copy number effects of the fusion on a plasmid and titration of the Fur repressor were avoided. *lacZ* fusions of the regions bp 77–877 and bp 640–877 upstream of *mchX* in both orientations were repressed by iron and strongly derepressed under low iron (SIP1004, SIP1005, SIP1003 and SIP1010). Usually only one orientation leads to full regulation of a promoter fused to the *lacZ* gene. To show that the iron regulation is mediated by Fur, *fur* mutations of the strains were constructed (SIP1011, SIP1013, SIP1014 and SIP1015). The β -galactosidase in these strains was strongly derepressed, independent of the iron concentration. Transformation with a *fur* plasmid (pSP61/18) restored the iron regulation, which indicated that Fur is responsible for this regulation.

Competitive growth of *E. coli* DSM 6601 with *E. coli* K-12

It has been shown that *E. coli* DSM 6601 is able to compete efficiently with certain enterobacteria in a co-culture on liquid media. We wanted to test whether the observed dominance of strain DSM 6601 is influenced by the iron-dependent microcin production. The microcin-producing strain DSM 6601 was compared to strain SK22D, in which *mchF* (region encoding the N-terminal part of the protein),

mchE, *mchD* and *mchC* (region encoding the C-terminal part of the protein) are deleted. *E. coli* K-12 WL311 Δlac was used as the competing strain. Each strain of each pair (DSM 6601 and WL311; SK22D and WL311) was inoculated at approximately 1×10^8 cells ml⁻¹ in iron-rich TY medium or in TY medium containing desferal, added to create low-iron conditions. At appropriate times during growth, an aliquot of each tube was plated on MacConkey/lactose agar plates, on which strains DSM 6601 and SK22D formed red colonies, while strain WL311 formed white colonies. In TY medium, strain DSM 6601 inhibited growth of strain WL311 by a factor of 10 (Fig. 2a). To create low-iron growth conditions, the siderophore desferal was added. Competitive growth of strains DSM 6601 and WL311 in TY medium containing 50 μ M desferal led to a strong suppression of growth of strain WL311. Already after 3 h, the number of c.f.u. of strain WL311 decreased, and after 7 h, the number of c.f.u. of strain WL311 dropped below 1×10^7 c.f.u. ml⁻¹ and was no longer detectable in our assay. In TY medium, strain SK22D grew as well as strain WL311 (Fig. 2c). In TY medium containing desferal, strain SK22D grew fivefold better than strain WL311 (Fig. 2d); however, its growth was clearly lower than observed for DSM 6601 with WL311.

DISCUSSION

E. coli DSM 6601 was one of the first strains isolated with the idea of using the strain as a competitor to inhibit growth of pathogenic bacteria in the gut. Since then, additional strains with suppressing activities against pathogenic strains have been isolated in various laboratories (Portrait *et al.*, 1999).

Successful competition of the non-pathogenic *E. coli* strain DSM 6601 with various micro-organisms, especially with pathogenic ones, had been claimed to be the major reason for its beneficial influence on the health of the gut. Here, it is demonstrated that strain DSM 6601 can outcompete an *E. coli* K-12 derivative strain; on low-iron medium, growth of *E. coli* K-12 was even more strongly suppressed. Less-efficient suppression was observed on the same low-iron medium by strain SK22D $\Delta mchDEF$, which is unable to export microcins. The production of microcins might indeed help strain DSM 6601 to compete with enterobacteria. However, also without microcin production, there is a preponderance of strains DSM 6601 and SK22D over strain WL311 in competition experiments; the reason might lie in the higher fitness of strain DSM 6601, which encodes, for example, the alternative iron-uptake systems and the corresponding biosynthesis genes for aerobactin, yersinia-bactin (Schubert *et al.*, 1999; Blum *et al.*, 1995) and salmochelin (K. Hantke & G. Winkelmann, unpublished).

Through comparisons of the known genome sequence of *E. coli* K-12 MG1655 with sequences of other *E. coli* strains, it becomes evident that since the isolation of *E. coli* K-12 in 1924, several unintended mutations have occurred that have certainly lowered its fitness, at least for life in the

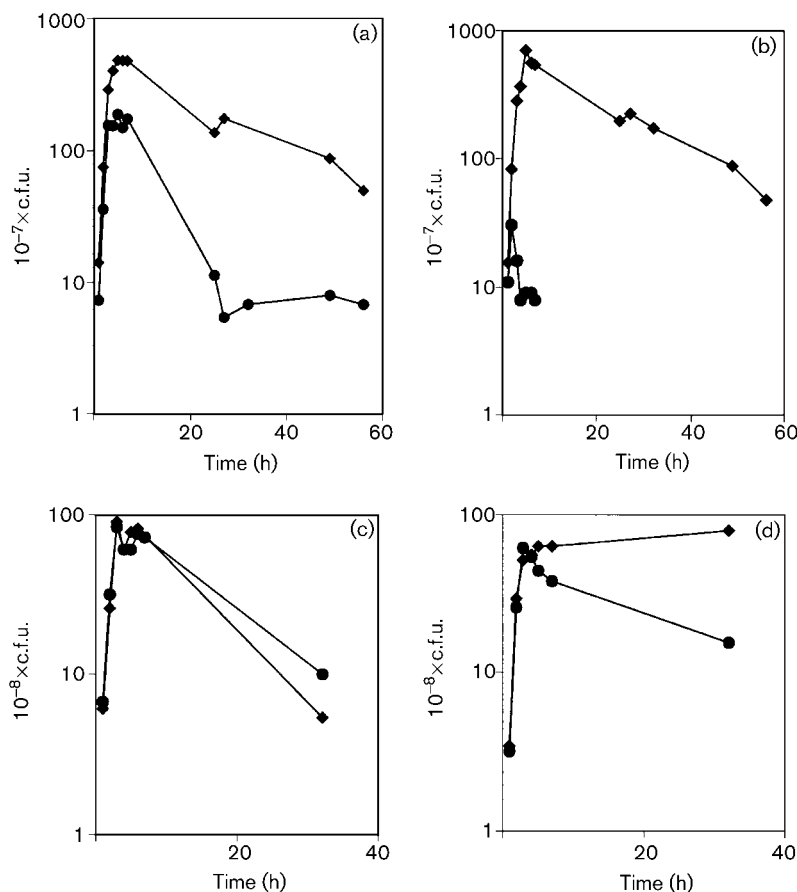


Fig. 2. (a, b) Competitive growth of strains DSM 6601 (◆) and WL311 (●) in TY medium (a) and in TY medium containing 0.05 mM desferal (b). (c, d) Competitive growth of strains SK22D (◆) and WL311 (●) in TY medium (c) and in TY medium containing 0.05 mM desferal (d).

gut, which might also explain its inferiority in our experiments. The main objective was to determine whether microcin production under iron restriction could help strain DSM 6601 to succeed in competition with other *E. coli* strains or other enterobacteria sensitive to at least one of the two microcins.

It is interesting to note that the microcin M/H47 determinant has been found independently five times in *E. coli* strains CA46 (colicin G producer), CA58 (colicin H producer), H47, DSM 6601 and CFT073 (where microcin production remains to be shown). In addition, remnants of this determinant have been found in the uropathogenic *E. coli* strain 536, in which there is a sequence nearly identical to *mchF' mcmIA mcmM* (Fig. 1) on the pathogenicity island III, on which also the *iro* gene cluster is found (Dobrindt *et al.*, 2001). In *E. coli* EDL933 O157:H7, the left side of the determinant with *mcmL* is found, followed by part of an IS1 element and the *mcmM* gene starting at bp 291 289 (GenBank accession number AP002554.1); this might indicate that the main part of the microcin determinant has been deleted.

The microcins produced by strains DSM 6601, CA46 (colicin G producer) and CA58 (colicin H producer), and transformants carrying pHM1 or pEX4, seem to be very similar or even identical. FepA might be a major receptor for

microcin H47, although Cir or Fiu contributed substantially to the sensitivity of the mutants (Table 4). Only the triple mutant *fepA cir fiu* was completely resistant. *E. coli* RYC1000(pHM1) produced both microcins H47 and M at levels higher than observed with *E. coli* RYC1000(pEX4). Microcin M S92C utilized mainly the receptors Fiu and FepA, while high microcin activities were necessary to kill a strain containing only Cir as a receptor.

E. coli DSM 6601 showed only a residual activity, which can be explained by the loss of *mcmL/mcmK*. It remains to be seen whether it will be possible to demonstrate that the original strain had an intact microcin H47/microcin M determinant. The residual microcin activity might possibly come from complementation by the very similar genes *iroB* and *iroD* in the *iro* gene cluster (Table 4).

Microcin sequences of the colicin V family are shown in Fig. 3. Bacteriocins, especially colicins, have a modular structure consisting of different combinations of receptor specificities and activity domains that arose through evolution (Braun *et al.*, 2002). Such a mosaic structure of short conserved regions is also observed with microcin M; up to amino acid residue 68 there is a 43 % identity to colicin V, while the last 17 C-terminal amino acid residues have 65–70 % identity to the C-terminus of microcins H47 and E492. It is tempting to speculate that this is the region where

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