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Growth Control of Small-Colony Variants by Genetic Regulation of the Hemin Uptake System

Andreas Roggenkamp,* Harald Hoffmann, and Mathias W. Hornef

Max von Pettenkofer Institute for Hygiene and Medical Microbiology, Department of Bacteriology, University Hospital Groβhadern, Ludwig-Maximilian University, D-81377 Munich, Germany

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Small-colony variants (SCVs) are slow-growing variants of human bacterial pathogens. They are associated with chronic persistent infections, and their biochemical identification and antimicrobial treatment are impaired by altered metabolic properties. To contribute to the understanding of SCV-mediated infections, we analyzed a clinical SCV isolate derived from a chronic prosthetic hip infection. A sequence analysis of housekeeping genes identified an *Enterobacter hormaechei*-like organism. The SCV phenotype, with growth as microcolonies, was caused by a block within the heme biosynthesis pathway through deletion of the *hemB* locus, as shown by hybridization and complementation experiments. At a low frequency, large-colony variants (LCVs) arose that were dependent on exogenous hemin. To investigate this phenomenon, we cloned and sequenced the 5.8-kb hemin uptake system, denoted *ehu*. Gene expression analysis indicated regulation of this locus in wild-type bacteria by the global iron regulator Fur. Inactivation of Fur in LCVs caused the derepression of *ehu* expression and facilitated bacterial growth. Genetic alterations of the *fur* locus in LCVs were identified as insertions of IS*1A* elements and point mutations. In contrast, SCVs could utilize exogenous hemin only in the absence of iron. Thus, we provide the first molecular characterization of the growth properties of a clinical SCV isolate, which may help to improve the diagnostic and therapeutic management of patients with chronic persistent infections.

The isolation of small-colony variants (SCVs) from clinical specimens has been reported since the beginning of the last century (16). These growth-deficient variants are formed by a wide range of gram-positive and gram-negative bacteria, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Although SCVs have repeatedly been associated with persistent and recurrent infections of the skeletal system, the heart, the lung, and indwelling medical devices, their precise role in the pathogenesis of infectious diseases has not been defined (13).

SCVs are characterized by reduced growth on routine culture media, with colony sizes of <1/10 the size appropriate for the species. Biochemical differentiation is impaired by decreases in metabolic properties, such as the fermentation of carbohydrates. Enhanced resistance against various antibiotics, particularly aminoglycosides, complicates antibiotic therapy and medical management. A deficiency in aerobic respiration is indicated by auxotrophy for hemin, thiamine, or menadione. SCV strains are characterized by an unstable growth behavior. Rapidly growing subpopulations, so-called revertants or large-colony variants (LCVs), arise spontaneously at low frequencies (24).

Despite a considerable number of reports about the isolation of SCVs from clinical specimens, the underlying molecular mechanisms of the altered growth properties have not been investigated. Members of our laboratory recently presented the first detailed molecular characterization of a clinical SCV strain isolated from a chronic prosthetic hip infection (29). The SCV phenotype of the gram-negative isolate Z-2376 was due to a deletion of the *hemB* gene. *hemB* codes for the single pathway enzyme, porphobilinogen synthase, that is required for heme biosynthesis (2). Heme derivatives are key components of the electron transfer apparatus, and heme deficiency causes a broad range of metabolic disturbances and a lack of intracellular energy. In fact, in vitro mutations in heme biosynthesis genes have been reported to generate strains with reduced growth rates, similar to the SCV phenotype (18, 34).

The examined clinical isolate, Z-2376, showed an unstable growth behavior, forming two colony types (29). On rich, hemin-containing agar plates, the majority of the strain grew as microcolonies (SCVs, with <0.1-mm diameters after 48 h at 37°C). Between these, large colonies (LCVs, with >1.5-mm diameters after 48 h at 37°C) appeared spontaneously with a frequency of 2×10^{-3} (Fig. 1). In contrast, both colony types grew as microcolonies on hemin-free agar plates. Although the emergence of fast-growing subpopulations among SCVs has been previously noted, the underlying functional mechanisms and the clinical importance are unknown (24). For the present study, we investigated this phenomenon and identified derepression of the global iron regulator Fur and an enhanced uptake of exogenous hemin by the hemin uptake system *ehu* to compensate for the heme biosynthesis defect.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Z-2376 was isolated from a chronic prosthetic hip infection (29). The type strain of *Enterobacter hormaechei* ATCC 49162 was purchased from the American Type Culture Collection. *Escherichia coli* K-12 strain DH5α was used for the cloning of *hemB*. *E. coli* strain W3110 was used as a Fur-proficient strain, and its *fur*-negative derivative H1673 was used as an *E. coli fur* mutant (33). The *E. coli* strain TOP10 was obtained from Invitrogen

* Corresponding author. Mailing address: Max von Pettenkofer Institute for Hygiene and Medical Microbiology, Department of Bacteriology, University Hospital Groβhadern, Ludwig-Maximilian University, Marchioninistr. 15, D-81377 Munich, Germany. Phone: 49-089-2180 78202. Fax: 49-089-2180 78207. E-mail: Andreas.Roggenkamp@med.uni-muenchen.de.

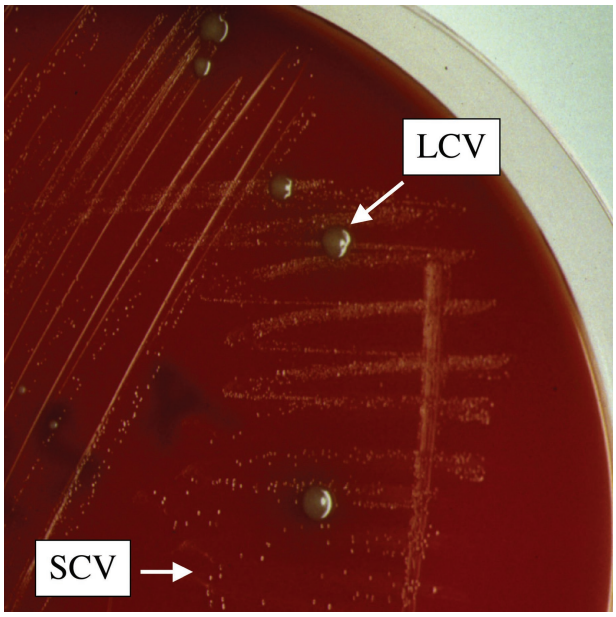


FIG. 1. Growth morphology of Z-2376. Z-2376 was grown on a Columbia blood agar plate for 48 h at 37°C. The majority of clones formed typical microcolonies characteristic for SCVs. A small subpopulation of Z-2376 grew as large colonies (LCVs).

(Karlsruhe, Germany). *E. coli* strains were routinely grown in Luria-Bertani medium, and strains Z-2376 and DH5α$_{hemB}$ were grown on Columbia blood agar plates or in brain heart infusion broth (BHI; Oxoid, Wesel, Germany). Nutrient broth (NB) supplemented with 0.25 mM 2,2′-dipyridyl (Sigma-Aldrich, Taufkirchen, Germany) (NBD) was used to generate low-iron growth conditions. Hemin (Sigma-Aldrich) was dissolved at a concentration of 25 mM in 20 mM NaOH. Antibiotic selection was performed with tetracycline (20 μg/ml) and ampicillin (100 μg/ml).

Selection of a heme biosynthesis-deficient mutant. The exposure of bacteria to aminoglycosides at or above their MICs was used to select for respiration-deficient mutants (Res$^-$) (14, 31). Res$^-$ mutants are unable to produce a proton gradient across the cytoplasmic membrane, which is a prerequisite for the uptake of aminoglycosides into bacterial cells. Most of the Res$^-$ mutants carried defects in heme biosynthesis genes (18). For generation of a mutant deficient in heme biosynthesis, *E. coli* DH5α was exposed to 8 μg of gentamicin/ml. The resulting microcolonies were analyzed by PCR and DNA-DNA hybridization to verify the presence or absence of the genes involved in the biosynthesis of heme. One mutant harboring a complete deletion of the *hemB* gene was chosen for further experiments and was named DH5α$_{hemB}$.

Molecular biological procedures. Plasmids and cosmids were isolated with NucleoBond anion-exchange columns (Macherey-Nagel, Düren, Germany). Chromosomal DNA was prepared by use of proteinase K and hexadecyltrimethylammonium bromide (Sigma-Aldrich) according to the protocol of Rahn et al. (27). Standard procedures were used for PCR, ligation, electroporation, transformation, and analysis of DNA and RNA (1). For cloning procedures, the proofreading *Pfu* DNA polymerase was used (Stratagene, La Jolla, Calif.). For DNA-DNA hybridization experiments, probes were generated and labeled with digitoxin-conjugated dUTP (Roche Diagnostics GmbH, Mannheim, Germany) by PCR. DNA sequencing was performed by the dideoxynucleotide chain termination method in an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, Calif.). The *ehu* operon and the *fur* gene were sequenced by primer walking in both directions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis were performed as described previously (28).

RT-PCR analysis. Reverse transcription (RT)-PCR was performed with the Access Quick RT-PCR system (Promega GmbH, Mannheim, Germany) and the primers HR-361 (5′-GATCCGGCGCTGATCAACG-3′) and HR-1108 (5′-TCTCATCCTGCAACCAGCC-3′) for *ehuA*. Amplification of the housekeeping gene *hsp60* (accession no. AB008137) with the primers HSP60-1 (5′-GGTAGAAGAAGGCGTGGTTGC-3′) and HSP60-2 (5′-ATGCATTCGGTGGTGATCATCAG-3′) was used as a control.

Identification and characterization of the hemin uptake locus of Z-2376. Chromosomal DNA was isolated from an LCV of Z-2376, partially digested with Sau3A, ligated into the BamHI site of the cosmid vector pLAFR2 (6), and packed in vitro (Stratagene). DH5α$_{hemB}$ was infected with recombinant phages and screened for tetracycline resistance and rapid growth in the presence of 8 μM hemin. Four independent colonies were isolated and denoted DH5α$_{hemB}$ pHR1 to pHR4. A restriction fragment analysis of the cosmids pHR1 to pHR4 showed identical patterns with three different enzymes.

Cloning of the *hemB* gene. For complementation of the *hemB*-deficient clinical isolate Z-2376 in *trans*, the *hemB* gene of DH5α was amplified as a 1.37-kb fragment by use of the primers HemB-1 (5′-GGTTGGATCCTTGGGGATAAACCG-3′) and HemB-2 (5′-GTATGGATCCTATGAATATGCAACAAAG-3′). Both primers include BamHI sites at their 5′ ends. The amplicon was digested with BamHI, ligated into the broad-host-range vector pSUP102, generating pGH1, and verified by sequencing (8).

Characterization of the *fur* locus in Z-2376. The *fur* gene of the clinical isolate Z-2376 was amplified by use of the following primers deduced from conserved regions of the *fur* genes of *E. coli* (accession no. D90707.1), *Klebsiella pneumoniae* (accession no. L23871.1), and *Salmonella enterica* serovar Typhimurium (accession no. AE008728.1): FldA-1 (5′-GAAGAGATYGAYTTCAAYGGCAAA-3′) and Fur-1 (5′-CAATNTCTTCACCNATATCGATCAG-3′) or Fur-2 (5′-GGTGYGATGTATGACCTGAAAAA-3′). Primers FldA-EH (5′-GACGATATCCTTAACGCCTGA-3′) and Fur2-EH (5′-CCTTCGGCACAGTGACCGTA-3′) were used for specific amplification of *fur* from Z-2376. IS*1A* PCR was done with the primers IS-1-76 (5′-CTGTCCCTCCTGTTCAGCTA-3′) and IS-1-711 (5′-GTCATGCAGCTCCACCGATT-3′), deduced from a known sequence (accession no. X52534).

EhuA antiserum preparation. For preparation of a rabbit antiserum against EhuA, a gene fragment of *ehuA* representing bp 643 to 1529 of the coding sequence was amplified by use of primers HRexT-1 (5′-CACCAGGGCGCCAAACGACGAAT-3′) and HRexT-2 (5′-CGGTAGTGGAGATGTAATCTT-3′), ligated into pET100, and transformed into *E. coli* strain TOP10 (Invitrogen). The resulting construct was verified by sequencing and denoted pETEhuA-1. Plasmid pETEhuA-1 was transformed into *E. coli* BL21 Star (Invitrogen), and gene expression was induced with 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 6 h at 37°C. The 33-kDa recombinant protein (rEhuA) was purified under denaturing conditions as recommended by the manufacturer. A 2-month-old New Zealand White rabbit was immunized with 150 μg of rEhuA and complete adjuvant (ABM adjuvant; Sebak GmbH, Aidenbach, Germany), followed by three booster immunizations of 150 μg of rEhuA and incomplete adjuvant each time.

Reporter gene analysis. EhuA-luciferase reporter fusions were constructed by using pCJYE138-L, a recently described pACYC184 derivative (12). A 1-kb *ehuA* promoter fragment carrying the *ehuA* promoter and the first eight codons was generated by PCR amplification from Z-2376 LCV DNA with primers HR-P-1 (5′-TGTCCTAAGCTTGAGCTCTCCTGTCCGGTGGGCT-3′) and HR-P-2 (5′-GCAGAGGATCCAGACGCGGATTGCAGGTGTGG-3′). The gene fragment was cloned into pCJYE138-L, replacing the HindIII/BamHI fragment (*yopE* insert); the resulting construct was denoted pEhuA-Luc. Luciferase reporter gene assays were performed as described by Jacobi et al. (12). In brief, cells were cultured for 6 h and then harvested by centrifugation, resuspended in lysate buffer, and frozen at −20°C. Determination of the optical density of the culture and serial plating were performed to standardize the bacterial lysate. The reporter gene activity of the bacterial lysates was measured for 10 min with a charge-coupled device camera.

Nucleotide sequence accession numbers. The nucleotide sequences of the *ehu* operon (AJ538328), the *fur* gene (AJ539162), and the IS*1A* (AJ539161) element of the clinical isolate Z-2376 have been deposited in GenBank.

RESULTS

Biochemical and genetic differentiation of Z-2376. The biochemical properties of the clinical strain Z-2376 isolated from a chronic prosthetic hip infection were not indicative of any known species (29). Z-2376 was therefore presumptively identified previously as *E. coli* based on its high level of 16S ribosomal DNA sequence similarity (>99%) (accession no. Z83204.1). Since the low reliability of 16S ribosomal DNA sequencing for the differentiation of *Enterobacteriaceae* has been demonstrated, we additionally performed sequencing of a

number of housekeeping genes (10, 32). Partial sequencing of *hsp60* and *rpoB* identified Z-2376 as a member of the genomic cluster X of the *Enterobacter cloacae* complex, most closely related to *E. hormaechei* (11; also data not shown).

Reversion of the growth defect of Z-2376 by complementation with the *hemB* gene of *E. coli*. DNA hybridization experiments had previously indicated that the *hemB* gene is deleted in the small and the large colony types of the clinical isolate Z-2376 (29). To verify that the deletion of *hemB* is responsible for the growth defect of Z-2376, we introduced the *hemB* gene of *E. coli* in the plasmid pGH1 into the SCV form of Z-2376. The resulting strain, Z-2376pGH1, exhibited the typical growth behavior of *Enterobacteriaceae* on MacConkey agar plates, with a colony size exceeding 1.5 mm after incubation at 37°C for 18 h, indicating successful restitution of the genetic defect. In addition, the metabolic properties of Z-2376pGH1 were analyzed by the API20E identification system of bio-Merieux Ltd. (Marcy l'Étoile, France). The resulting numerical code of Z-2376pGH1 (3305573, which is a very accurate identification of *Enterobacter* species) supported the genotypic identification of Z-2376 as a member of the *E. cloacae* complex. Thus, the resulting phenotype of the *hemB*-complemented SCV strain demonstrated that the deletion of *hemB* was indeed responsible for the growth deficiency of Z-2376 SCVs.

Cloning and sequencing of the hemin uptake locus *ehu* from Z-2376. Feeding experiments suggested that the colony size of Z-2376 LCVs was dependent on the presence of hemin (29). In contrast to Z-2376 SCVs, Z-2376 LCVs were able to utilize exogenous hemin, resulting in a larger colony size on hemin-containing BHI or Columbia blood agar plates (Fig. 1). Since the cell membranes of gram-negative bacteria are impermeable for heme, specific hemin uptake systems are required (7). However, *E. coli* K-12 has been shown to lack heme uptake systems and no such system has been described for members of the *E. cloacae* complex. In order to identify genes responsible for the utilization of hemin by Z-2376 LCVs and subsequently to identify the mechanism controlling the formation of LCVs in Z-2376, we constructed a Sau3A cosmid library of Z-2376 LCVs in an *E. coli hemB* mutant (DH5α_{hemB}) and screened them for growth on hemin-containing medium. Four independent clones exhibited hemin-dependent growth behavior, forming normally sized colonies (>1.5 mm) on BHI-hemin-agar plates but forming microcolonies (<0.1 mm) on non-supplemented growth medium. A restriction fragment analysis of the isolated cosmids using three different enzymes showed identical restriction patterns. Sequence analysis identified a previously unknown operon of 5.8 kb with homologies to reported hemin uptake loci in other gram-negative bacteria (Fig. 2). A 10-kb EcoRI/SphI fragment which encoded the complete operon and was sufficient to promote the growth of DH5α_{hemB} on hemin-containing agar was subcloned (data not shown).

The genetic organization of the hemin uptake locus of Z-2376 LCVs was denoted *ehu* (for *Enterobacter* hemin uptake). It revealed close homology to the *hem* operon of *Yersinia enterocolitica*. Also, the first protein encoded by this operon, denoted EhuA, exhibited the following conserved features of outer membrane hemin receptor proteins: (i) an N-terminal leader sequence of 29 amino acids (aa), (ii) a TonB box (aa 31 to 38), (iii) region V of Ton B-dependent proteins (aa 523 to 531), (iv) FRAP (aa 434 to 437) and NPNL (aa 462 to 465) motifs as well as the conserved histidine residues (aa 115 and 451) recently described by Bracken et al. (3), and (v) a C-terminal aromatic tryptophan residue required for proper incorporation into the outer membrane (15). Additionally, a putative Fur box (GATAATGATTATCATTGT) was found 296 to 313 bp upstream of the putative transcriptional start site of the operon, indicating iron-dependent regulation (4).

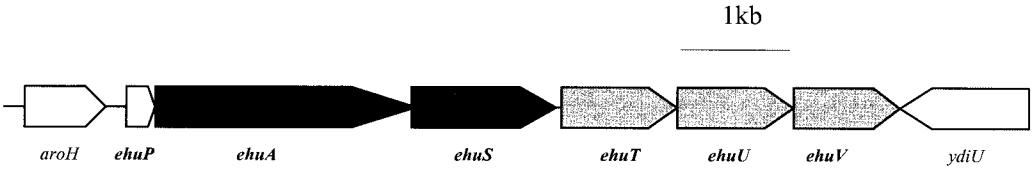
Subsequently, possible genetic differences between the hemin-utilizing LCV type of Z-2376, the SCV type of Z-2376, and the type strain of *E. hormaechei* were investigated. DNA probes recognizing a variety of different genes of the *ehu* operon hybridized specifically with chromosomal DNAs of the *E. hormaechei* type strain and both colony variants (SCVs and LCVs) of Z-2376 (data not shown). Also, sequencing of PCR products amplified from the *E. hormaechei* type strain and Z-2376 SCVs and LCVs showed that all three strains encoded the complete *ehu* hemin uptake system. Alterations of the DNA sequence, particularly within the promoter region of *ehuA*, could not be identified. Thus, we identified and characterized the *Enterobacter* hemin uptake system *ehu*. However, no genetic differences were detected between the type strain and SCV subpopulations.

***ehu* is regulated by Fur in an iron-dependent fashion.** Hemin uptake systems in *Enterobacteriaceae* are controlled by the global regulator Fur and are upregulated under conditions of low iron concentrations (4, 7, 9, 17). Fur binds as a transcriptional repressor to a consensus sequence know as the Fur box located upstream of all Fur-regulated genes. Similarly, a putative Fur box was identified in Z-2376 upstream of the first gene of the *ehu* operon, *ehuA*. To verify that genes of the *ehu* operon were regulated by Fur in an iron-dependent fashion, we constructed an EhuA-luciferase reporter fusion. Expression analysis was subsequently performed with Fur-positive *E. coli* W3110 and the Fur-deficient derivative H 1673 under iron-rich and iron-depleted conditions. As expected for a Fur-regulated gene, EhuA-luciferase was produced by the wild-type strain exclusively in iron-depleted medium (NBD) (54,097 ± 6,770 relative light units [mean ± standard deviation] versus 899 ± 101 in NB). In contrast, a high reporter gene activity was found for the *E. coli fur* mutant H 1673 regardless of the presence or absence of iron (81,002 ± 7,171 relative light units in NBD and 53,314 ± 7,964 in NB). Thus, the reporter gene analysis performed with *E. coli* suggested that the *ehu* operon is controlled by the global iron regulator Fur, leading to upregulation under conditions of low iron concentrations. The regulation of the *ehu* operon might differ in Z-2376. However, attempts to analyze *ehu* regulation in Z-2376 by genetic manipulations, e.g., the construction of a *fur* mutant, failed.

EhuA is dysregulated in both colony types of Z-2376. For confirmation of the iron-dependent expression of EhuA, an immunoblot analysis of whole bacterial cell lysates was performed using a polyclonal rabbit antiserum raised against EhuA. Analysis of the type strain of *E. hormaechei* and the *hemB*-complemented Z-2376pGH1 strain revealed that there is indeed induction of EhuA synthesis in the absence of iron (Fig. 3, lanes 3 and 4).

Whereas both colony types of Z-2376 encode the *ehu* hemin uptake system, only the LCV subtype of Z-2376 was able to utilize exogenous hemin. Accordingly, the production of EhuA in Z-2376 SCVs and LCVs in the presence or absence of iron

A



B

Protein (molecular weight)[a] (pI)[b]	Identity (%)	Similarity (%)
EhuP 62 aa (6736) (8.6)		
E. coli YdiE, putative	54	68
Y. enterocolitica HemP	48	53
EhuA (pfam00593) outer membrane hemin receptor, TonB dependent, 660 aa (72103)(4.8)		
S. dysenteriae ShuA	79	87
E. coli ChuA	79	87
Y. enterocolitica HemR	66	77
Y. pestis HmuR	66	79
EhuS hemin transport/degrading, cytoplasmic, 356 aa (39793)(5.8)		
S. dysenteriae ShuS	82	90
E. coli ChuS	82	90
Y. pestis HmuS	67	79
Y. enterocolitica HemS	65	79
EhuT (pfam 01497, 242 aa) periplasmic heme-binding protein, 272 aa (29110)(9.2)		
Y. pestis HmuT	51	66
Y. enterocolitica HemT	51	66
EhuU (pfam 01032) FecCD transport family, inner membrane, hemin permease, 330 aa (34768)(8.9)		
Y. enterocolitica HemU	47	57
Y. pestis HmuU	47	57
E. coli ChuU	45	58
S. dysenteriae ShuU	45	58
EhuV (pfam 00005) ABC transporter, inner membrane, ATP-binding, 272 aa (29110)(9.2)		
Y. enterocolitica HemV	53	64
Y. pestis HmuV	51	64
E. coli ChuV	46	62
S. dysenteriae ShuV	46	62

[a] calculated
[b] GCG software package

FIG. 2. Hemin uptake system *ehu* of Z-2376. (A) Genetic organization of the hemin uptake system *ehu* of Z-2376. (B) Comparison of the proteins encoded by the *ehu* locus with known related heme proteins. Accession numbers for proteins are as follows: *Y. enterocolitica* proteins, X68147 and X77867; *S. dysenteriae* proteins, U64516; *E. coli* proteins, U67920 and D90813; and *Y. pestis* proteins, U60647.

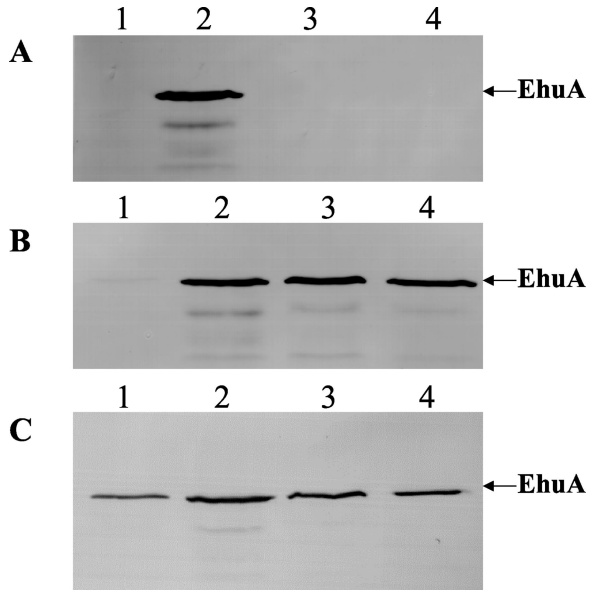


FIG. 3. Production of EhuA in Z-2376 SCVs and LCVs as well as *E. hormaechei*. An immunoblot analysis was performed with whole-cell lysates (30 μg) and a rabbit antiserum specific for EhuA (72.1 kDa). The different strains were grown at 37°C for 6 h in NB (A), iron-depleted NBD (B), or iron-depleted NBD containing 8 μM hemin (C). Lanes 1, Z-2376 SCVs; lanes 2, Z-2376 LCVs; lanes 3, Z-2376pGH1; lanes 4, *E. hormaechei* ATCC 49162.

was compared. Unexpectedly, Z-2376 LCVs were able to produce EhuA even in the presence of iron, indicating a derepressed expression of *ehuA*. In contrast, immunoblot analysis failed to detect any EhuA expression in Z-2376 SCVs, even under iron-depleted conditions (Fig. 3A and B). However, Z-2376 SCVs were able to produce EhuA in iron-depleted medium in the presence of hemin (Fig. 3C). Thus, in contrast to the derepressed expression of *ehuA* in Z-2376 LCVs, the Z-2376 SCVs produced EhuA in iron-depleted medium only in the presence of hemin.

Transcriptional and posttranscriptional regulation of EhuA. RT-PCR analysis was performed to investigate whether the lack of EhuA production in Z-2376 SCVs cultured in iron-depleted, hemin-free medium was a transcriptional or posttranscriptional phenomenon. As shown in Fig. 4, *ehuA* was transcribed in Z-2376 SCVs in iron-depleted medium independent of the presence or absence of hemin. The posttranscriptional stop of EhuA production in hemin-free medium was an unexpected finding. Notably, a direct influence of hemin on the regulation and expression of Fur-regulated genes has not previously been described for members of the *Enterobacteriaceae*.

Correlation between EhuA production and utilization of hemin in Z-2376. To analyze if the production of EhuA correlated with the utilization of exogenous hemin in Z-2376, we performed growth experiments in different culture media in the present or absence of hemin (Fig. 5). Corresponding to the size of the colonies on hemin-containing agar plates, Z-2376 LCVs, but not SCVs, utilized hemin in iron-containing media and multiplied to the same extent as the *E. hormaechei* type strain. In the absence of hemin, both colony types of Z-2376 were not able to grow in NB. In contrast, Z-2376 SCVs multiplied in iron-depleted medium in the presence of hemin, suggesting that the production of EhuA was functional, enabling the strain to utilize hemin under conditions of low iron concentrations. Attempts to inactivate *ehuA* in Z-2376 SCVs or LCVs by marker exchange using kanamycin- or chloramphenicol-resistant genes and temperature-sensitive suicide vectors failed due to a marked resistance of Z-2376 against transformation. Thus, in contrast to the iron-independent utilization of hemin by LCVs, Z-2376 SCVs were able to utilize hemin and to multiply comparably to an *Enterobacter* wild-type strain only under the appropriate conditions.

Cloning of the Z-2376 *fur* gene. Z-2376 LCVs were able to produce EhuA independent of the presence of iron, indicating a derepressed expression of *ehuA*. Therefore, we next investigated a possible mutational inactivation of the global iron regulator *fur* in Z-2376 LCVs. Since the sequence of the *Enterobacter fur* gene was unknown, the *fur* gene of Z-2376 SCVs was initially cloned and sequenced by using oligonucleotides deduced from conserved regions upstream and downstream of the *E. coli fur* gene. Gene products of the appropriate size could be amplified from Z-2376 SCVs. A DNA sequence analysis of the PCR products revealed one open reading frame (ORF), with 447 bp coding for 148 aa. The translated gene product showed 95% identity and 98% similarity to Fur of *E. coli*. The promoter region upstream of the ORF contained a putative Fur-binding site (64 to 91 bp upstream of the start codon) and a putative OxyR-binding site (215 to 263 bp upstream of the start codon). Both motifs were also present in the *E. coli fur* operon (35). These results strongly suggested that we amplified the *fur* gene of Z-2376 SCVs.

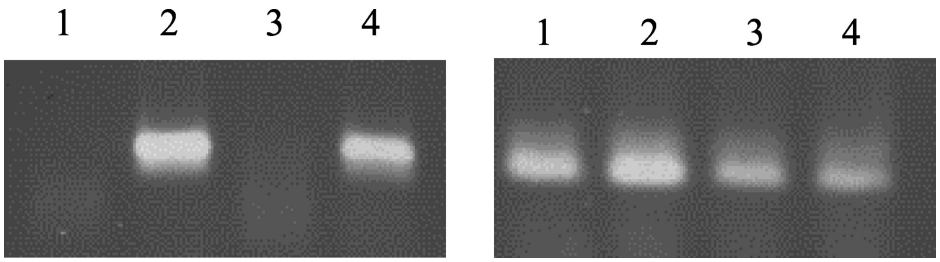


FIG. 4. Transcriptional expression of *ehuA* in Z-2376 SCVs. RNAs were prepared from Z-2376 SCVs incubated in different media for 2 h at 37°C. RT-PCR analyses of *ehuA* (741 nucleotides; left) and *hsp60* (350 nucleotides; right) are shown. Lanes 1, Z-2376 SCVs grown in NB; lanes 2, Z-2376 SCVs grown in iron-depleted NBD; lanes 3, Z-2376 SCVs grown in NB plus 8 μM hemin; lanes 4, Z-2376 SCVs grown in iron-depleted NBD plus 8 μM hemin.

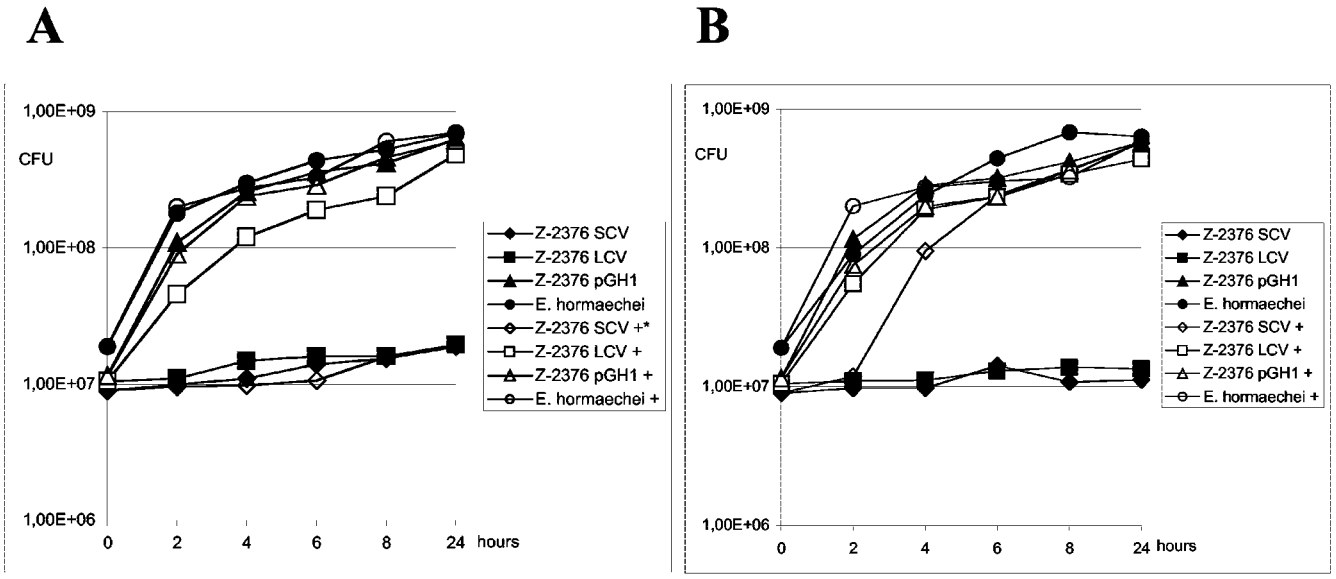


FIG. 5. Iron- and hemin-dependent growth of the different colony variants of Z-2376. Z-2376 SCVs and LCVs as well as *E. hormaechei* ATCC 49162 were grown in NB (A) or iron-depleted NBD (B). Medium supplementation with 8 μM hemin is indicated with "+" symbols. Bacterial growth was quantified by plating of serial dilutions on blood agar plates. The emergence of LCV variants in SCV cultures was excluded by repeated subcultures showing exclusively SCV-type colonies. The data represent one of three identical experiments.

Mutations of the *fur* gene are responsible for the formation of LCVs in Z-2376. To determine whether the genetic organization of the *fur* gene was altered in Z-2376 LCVs, we selected 12 independent colonies of Z-2376 showing the typical growth properties of LCVs. All isolates were analyzed by PCR using oligonucleotides located upstream and downstream of *fur*. Strikingly, in comparison to the *fur* locus of Z-2376 SCVs, the PCR products of nine Z-2376 LCV isolates were about 800 bp

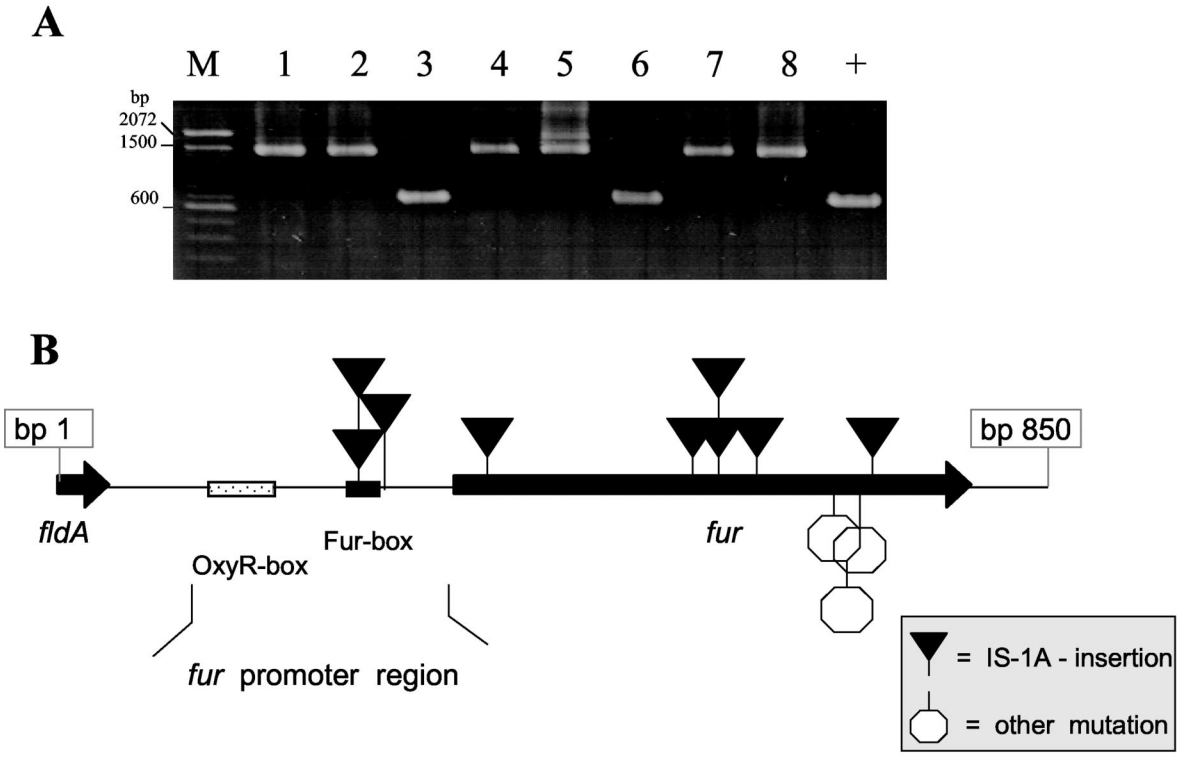


FIG. 6. PCR analysis of the *fur* locus of Z-2376 LCVs. (A) PCR analysis of the *fur* locus of different LCV-type colonies (LCVs 1 to 8) with primers FldA-EH and Fur2-EH. As a control, the result for Z-2376 SCVs is included (+). M, 100-bp DNA ladder. (B) Schematic drawing of all mutations within the *fur* locus identified in LCV-type colonies of Z-2376.

TABLE 1. Mutations within the *fur* gene of three different Z-2376 LCV isolates

Isolate no.	Nucleotide position of mutation	Description of mutation	Mutated amino acid
3	337	Duplication of 16 nucleotides plus C	Frameshift after aa 112
6	287	G→T	Cys 96→Phe96
11	294–296	Deletion of 3 nucleotides (GGT)	Lys 98 + Val 99→Asp

larger (Fig. 6A). DNA sequence analyses revealed that in each case the same insertion sequence (IS) element was inserted more or less randomly within the *fur* gene. Six isolates harbored the IS element within the coding sequence of *fur*, at positions 38, 174, 177 (two times), 233, and 334. Three other isolates harbored the IS element within the promoter region, 45 or 65 (two times) bp upstream of the start codon (for an overview, see Fig. 6B). The identified IS element exhibited 99% identity to known IS*1A* elements described for *E. coli* and other *Enterobacteriaceae* (accession no. AJ278144.1) (20). Similar to other described insertions of IS*1* elements, each IS*1A* *fur* insertion resulted in a duplication of 9 bp. However, no frameshift mutations within the two ORFs, *insA* and *insB*, of the IS*1* element, which have been described to increase the frequency of transposition, were detected (5). Subsequent hybridization experiments using labeled IS*1A* probes revealed that all nine LCV isolates with identified IS element insertions within the *fur* gene contained six IS*1A* copies in their chromosomes, whereas Z-2376 SCVs and Z-2376pGH1 harbored five (data not shown).

Additionally, we examined the underlying mechanism of *fur* inactivation of the remaining three LCV isolates, which did not show enhanced lengths of the *fur* locus. Careful sequence analysis revealed that there were indeed minor mutations of the *fur* gene in all three cases (Table 1). Besides a frameshift mutation leading to a premature stop, a point mutation as well as a short deletion responsible for an amino acid exchange within the Fur protein was found. Together, these results strongly suggest that an inactivation of *fur* was responsible for the derepression of the *ehu* locus in LCVs. Thus, whereas the deletion of *hemB* in SCVs facilitated growth only in the absence of iron, the additional inactivation of *fur* and derepression of *ehu* allowed LCVs of Z-2376 to internalize exogenous hemin independent of the presence of low iron concentrations.

DISCUSSION

Infections caused by SCV pathogens require a high level of attention and careful management from clinical laboratories and clinicians. SCVs often trigger long-lasting and recurrent infections and exhibit significant resistance to many antibiotics. Slow growth on standard culture media, altered biochemical properties, and colony polymorphisms frequently lead to insufficient or false identification in routine diagnostic laboratories. In addition, the emergence of SCVs may significantly contribute to the chronic persistent course of the infection. Very little is known about the effect of the reduced and unstable growth of SCVs on the pathogenesis of infection. It has been speculated that once a slow-growing SCV subpopulation of the original pathogen has been selected, the course of infection may slow down from a rapid, ongoing infection to a chronic, persistent one (13, 25, 26). Thus, the quiescent state of SCV isolates may trigger therapy-resistant chronic infections. In contrast, the appearance of more rapidly growing LCVs may be the reason for the recrudescence of SCV-mediated infections.

Here we present the first characterization of the underlying molecular mechanisms that lead to the development of slow-growing SCVs as well as the reemergence of rapidly proliferating LCVs derived from a clinical isolate of a chronic prosthetic hip infection. Our description also illustrates the typical problems that arise with routine laboratory biochemical and sequence-based identification techniques. Only sequencing of several housekeeping genes revealed that the SCV strain Z-2376 was an *E. hormaechei*-like organism and a member of the genomic cluster X of the *E. cloacae* complex. Therefore, this report adds another organism to the list of possible SCV-forming strains and stresses the general potential of this ill-defined phenomenon.

DNA hybridization and complementation experiments demonstrated that the deletion of the *hemB* gene was responsible for the reduced growth rate of SCV Z-2376 (29). HemB catalyzes an essential step in the heme biosynthesis pathway. Hemin itself is required as a cofactor of a variety of important enzymes, such as catalase, and of respiratory cytochromes. Therefore, the lack of heme biosynthesis results in a pleiotropic phenotype with (i) an incapability of generating energy from proton motive force, (ii) a lack of energy-dependent transport across the bacterial membrane, and (iii) enhanced susceptibility to oxidative stress (19). Heme biosynthesis mutants grow normally under anaerobic conditions; however, in an aerobic atmosphere, these mutants exhibit a distinct growth deficiency similar to the phenotype of SCVs (34).

At a low frequency, SCVs form fast-growing subpopulations, so-called revertants or LCVs. A careful analysis of this phenomenon led to the identification of the hemin uptake system *ehu*, the first hemin uptake system described for *Enterobacter* sp. The *ehu* operon was inserted between *aroH* and *ydiU* and exhibited the highest homology to the hemin uptake system *hem* of *Y. enterocolitica*. Other yet unknown hemin uptake systems may also contribute to the utilization of exogenous hemin in Z-2376. Multiple hemin uptake systems have been described for other gram-negative bacteria, such as *Yersinia* spp., *Vibrio cholerae*, and *P. aeruginosa* (21, 23, 30). However, hybridization experiments using low-stringency conditions and probes for *ehuA* or the alternative hemin uptake system of *Yersinia* spp. failed to detect a second hemin uptake system in Z-2376 (data not shown).

Similar to homologous hemin uptake systems in other gram-negative bacteria, *ehu* was regulated by the global repressor Fur in an iron-dependent manner. Fur is known as an important regulator, controlling more than 90 genes in *E. coli*, including a set of virulence factors (9). Derepression of *ehu* by the inactivation of Fur in LCV subpopulations was identified to facilitate normal growth on hemin-containing media. Interestingly, *fur* mutants have not been associated with an enhanced pathogenic potential so far. Spontaneous inactivation of Fur most often occurred by the insertion of IS*1A* elements. Phase

variation due to IS insertions has been reported for many genera (18, 36), but IS insertions have never been described for *fur*. IS*1A* insertions in *fur* appeared to be stable, since a transition from the LCV to the SCV phenotype was not observed. In addition to IS*1A* insertions, other mutations within the *fur* gene were identified that impaired the regulatory functioning of Fur. However, the formation of LCVs in other SCV strains could be caused by other mechanisms. For example, Lewis et al. described unstable *E. coli hemB* mutants that emerged in vitro and harbored a precise excision of the IS*2* element from *hemB* (18).

To summarize the results of the in vitro experiments, the growth of SCVs and LCVs of Z-2376 was dependent on the presence of hemin and iron. Due to the deletion of *hemB*, both types of colony variants were defective in heme biosynthesis, and due to the presence of *ehu*, both compensated for the defect by utilizing exogenous hemin. In the absence of hemin, the growth of both types of colony variants was strongly reduced irrespective of the presence of iron. The presence of hemin and iron promoted the rapid growth of LCVs but not SCVs. LCVs carried second mutations in *fur*, resulting in the derepression of the heme uptake system *ehu*, the production of the hemin receptor EhuA, the utilization of hemin, and the acceleration of growth. In contrast, the *fur* gene remained unaltered in SCVs, and in the presence of iron, Fur repressed the transcription of *ehu*, EhuA was not produced, and hemin was not utilized, resulting in reduced growth and the formation of microcolonies. In iron-depleted, hemin-containing medium, both colony types multiplied equally well. For LCVs, *ehu* remained derepressed and hemin was utilized. For SCVs, the absence of iron resulted in the transcription of *ehu* and subsequently in the utilization of hemin.

Interestingly, EhuA was only produced by SCVs under iron-deficient conditions in the presence of hemin. A direct influence of hemin on the expression of hemin uptake systems in *Enterobacteriaceae* has not previously been described. However, since *ehu* was regulated independently of hemin in the *E. hormaechei* type strain, the hemin-dependent production of Ehu in Z-2376 SCVs might have been due to a lack of energy in this heme biosynthesis-negative mutant.

The in vitro growth behavior of Z-2376 may be of relevance for in vivo conditions. Importantly, iron is restricted inside the mammalian host and hemin may be available for a pathogen, particularly at the site of infection and inflammation. Thus, SCVs may not persist in a quiescent state inside the host as recently postulated. The expression of the hemin uptake system compensated for the chromosomal deletion of *hemB* in vivo. Fur inactivation and LCV formation of Z-2376 occurred spontaneously at a low frequency under in vitro conditions. The occurrence of a *hemB fur* double mutant might be advantageous under microaerophilic conditions with variable iron and hemin concentrations. To investigate this question, we are currently developing an animal model. This will also allow for the analysis of other important features, such as the production of virulence factors (e.g., capsular polysaccharides and hemolysin), of SCV strains. A recent report by Musher et al. demonstrated a similar pathogenic potential of gentamicin-resistant SCVs compared to their parental strain (22). However, this study used in vitro-generated SCVs with an unknown molecular background.

In conclusion, we have presented the first molecular characterization of the mechanisms that lead to the development of SCVs and demonstrated a critical role for the regulation of the hemin uptake system in the emergence of fast-growing subpopulations. These results advance our understanding of the mechanisms that contribute to the development of chronic persistent infections and will help us to develop new diagnostic and therapeutic strategies to improve the clinical management of infected patients.

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