The Biofilm Inhibitor Carolacton Enters Gram-Negative Cells: Studies Using a TolC-Deficient Strain of *Escherichia coli*

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**ABSTRACT** The myxobacterial secondary metabolite carolacton inhibits growth of *Streptococcus pneumoniae* and kills biofilm cells of the caries- and endocarditis-associated pathogen *Streptococcus mutans* at nanomolar concentrations. Here, we studied the response to carolacton of an *Escherichia coli* strain that lacked the outer membrane protein TolC. Whole-genome sequencing of the laboratory *E. coli* strain TolC revealed the integration of an insertion element, IS5, at the tolC locus and a close phylogenetic relationship to the ancient *E. coli* K-12. We demonstrated via transcriptome sequencing (RNA-seq) and determination of MIC values that carolacton penetrates the phospholipid bilayer of the Gram-negative cell envelope and inhibits growth of *E. coli* TolC at similar concentrations as for streptococci. This inhibition is completely lost for a C-9 (R) epimer of carolacton, a derivative with an inverted stereocenter at carbon atom 9 ([S] → (R)) as the sole difference from the native molecule, which is also inactive in *S. pneumoniae* and *S. mutans*, suggesting a specific interaction of native carolacton with a conserved cellular target present in bacterial phyla as distantly related as *Firmicutes* and *Proteobacteria*. The efflux pump inhibitor (EPI) phenylalanine arginine β-naphthylamide (PAβN), which specifically inhibits AcrAB-TolC, renders *E. coli* susceptible to carolacton. Our data indicate that carolacton has potential for use in antimicrobial chemotherapy against Gram-negative bacteria, as a single drug or in combination with EPIs. Strain *E. coli* TolC has been deposited at the DSMZ; together with the associated RNA-seq data and MIC values, it can be used as a reference during future screenings for novel bioactive compounds.

**IMPORTANCE** The emergence of pathogens resistant against most or all of the antibiotics currently used in human therapy is a global threat, and therefore the search for antimicrobials with novel targets and modes of action is of utmost importance. The myxobacterial secondary metabolite carolacton had previously been shown to inhibit biofilm formation and growth of streptococci. Here, we investigated if carolacton could act against Gram-negative bacteria, which are difficult targets because of their double-layered cytoplasmic envelope. We found that the model organism *Escherichia coli* is susceptible to carolacton, similar to the Gram-positive *Streptococcus pneumoniae*, if its multidrug efflux system AcrAB-TolC is either inactivated genetically, by disruption of the tolC gene, or physiologically by coadministering an efflux pump inhibitor. A carolacton epimer that has a different steric configuration at car-
bon atom 9 is completely inactive, suggesting that carolacton may interact with the same molecular target in both Gram-positive and Gram-negative bacteria.

**KEYWORDS** Gram-negative bacteria, antimicrobial activity, antimicrobial agents, carolacton, drug efflux, drug resistance mechanisms, efflux pumps, gene sequencing, genome analysis

The identification of novel compounds for antimicrobial chemotherapy is becoming increasingly difficult (1). This is especially true for compounds targeting Gram-negative bacteria, for two main reasons: first, the second outer plasma membrane of Gram-negative organisms acts as a potent barrier and restricts the entry of hydrophilic extracellular substances, such as antibiotics, into the cell (2); second, the multidrug resistance (MDR) efflux systems present in many Gram-negative bacteria provide intrinsic resistance against antibiotics (3). The primary function of MDR efflux systems is the removal of toxins and bile acids from the cytoplasm, which is important for infectivity and virulence (4). MDR facilitated by extrusion of antibiotics has become a serious problem in the treatment of infections by, e.g., *Escherichia coli* (5), *Klebsiella pneumoniae* (6), *Pseudomonas aeruginosa* (7), and *Salmonella enterica* (8).

Proton-dependent tripartite envelope translocase systems (TETS) are widely distributed MDR efflux systems which have been studied extensively in *E. coli* and *P. aeruginosa*. TETS characteristically consist of an MDR pump, a membrane fusion protein (MFP), and an outer membrane factor (OMF) (9). In *E. coli*, MDR pumps of the resistance-nodulation-division (RND) family are key contributors to intrinsic antibiotic resistance (10). The genome of *E. coli* includes six genes for MDR pumps of the RND family (*acrB, acrC, yhiU, acrD, yegN*, and *yegO*) and seven genes for MFPs (*acrA, acrE, yhiV, yegM, emrA, emrK*, and *ybjY*) (11). As the third component of tripartite efflux systems, *E. coli* possesses four genes encoding OMF proteins, *tolC, mdtP, mdtQ*, and *cusC*, which are essential for a functional RND pump (e.g., *AcrA-AcrB-TolC*) (11). Among all OMF proteins of *E. coli*, TolC appears to be the major facilitator for extrusion of antibiotics and small molecules through the outer membrane (11, 12). In particular, the AcrAB-TolC tripartite efflux system is of great scientific interest, since it is constitutively expressed, has a broad substrate specificity, is found in a wide variety of clinically relevant Gram-negative pathogens (e.g., *P. aeruginosa, S. enterica*, and *Klebsiella spp.*), and contributes to MDR (4).

Therefore, efflux pump inhibitors (EPIs) can be important for the discovery of novel antibiotics (13), and they can be applied in combination with current antibiotics to overcome extrusion by MDR efflux systems (13, 14). Among them, the peptidomimetic EPI phenylalanine arginine β-naphthylamide (PAβN; MC-2077110) (15) was found to specifically block the AcrAB- and AcrEF-based MDR efflux systems in *E. coli*, which are both dependent on TolC as the OMF (16). On the other hand, bacterial strains with defects in MDR efflux systems are often used as sensitive indicators for antimicrobial activity (17). TolC mutants of *E. coli*, for example, are hypersensitive to 19 of 22 antibiotics tested (12).

The screening of libraries of natural secondary metabolites holds great promise for the discovery of novel antimicrobial compounds (18). During such screenings, the myxobacterial macroclide ketocarbonic acid carolacton was identified as a biofilm inhibitor (19, 20). Its activity against clinically relevant streptococci was later analyzed in great detail (20–24). The exact molecular target of carolacton remains unknown, but the complete loss of biological activity of a carolacton epimer at C-9 ([S] → (R)] (epi-carolacton) in *Streptococcus mutans* biofilms and planktonically growing *Streptococcus pneumoniae* cells (22, 24) suggests an interaction of carolacton with a conserved cellular target (24).

Carolacton is inactive against *E. coli* (MIC, >40 µg/ml), but strong growth inhibition was found when a laboratory *E. coli* strain recorded as lacking a functional copy of the OMF TolC (*E. coli* TolC) was treated with carolacton (MIC, 0.06 µg/ml) (19). These data suggested that carolacton might be able to pass through the Gram-
Inhibitory Activity of Carolacton against *E. coli*
negative cell envelope and that the lack of sensitivity of wild-type *E. coli* to carolacton is due to export from the cell by TolC-mediated efflux. However, mutations in TolC can have different effects on substrate export, and there have even been reports that a misassembled TolC protein may result in an open channel which allows influx of antibiotics into the cell, resulting in an increased sensitivity (25). The TolC-deficient strain used in our screenings has been propagated as a glycerol stock in laboratories since at least 1980 (B. Kunze, personal communication), and so far it has not been characterized genetically. Over a period of 37 years, massive genetic changes could have occurred (26). Moreover, although TolC-deficient strains are used by many laboratories, they were constructed with different methods and in different genetic backgrounds (25, 27, 28), making it hard to compare results. We here determined the genome sequence of *E. coli* TolC with high resolution by using a combination of PacBio and Illumina sequencing. With these methods, an insertion of a natural transposon at the tolC locus was identified, and genetic changes were recorded that had occurred in this strain in comparison to its closest relative, which was identified as *E. coli* K-12 MG1655 (NZ_CP014225.1). We determined MICs for *E. coli* K-12 MG1655 and *E. coli* TolC and deposited *E. coli* TolC with the DSMZ as a tool and reference for future studies. We then studied the influence of carolacton on *E. coli* TolC by using transcriptome sequencing (RNA-seq), the carolacton C-9 (*R*) epimer, and the EPI PAβN. The data clearly showed that carolacton easily penetrates the Gram-negative cell envelope. Once inside the cell, it inhibits *E. coli* at similar concentrations as for streptococci, suggesting that the molecular target of carolacton is highly conserved and might be highly similar even in distantly related bacterial phyla, such as *Firmicutes* and *Proteobacteria*. The export of carolacton from the cell can be overcome by blocking the AcrAB-TolC efflux complex with the EPI PAβN. This finding highlights the potential use of carolacton in combinatorial treatment with EPIs.

**RESULTS**

*E. coli* TolC is an ancient natural derivative of *E. coli* K-12 and is closely related to K-12 MG1655. PacBio single-molecule real-time (SMRT) sequencing and Illumina MiSeq short-read sequencing were combined to obtain a high-quality genome sequence of *E. coli* TolC. By Illumina MiSeq sequencing, 2,623,454 reads were obtained, totaling ~656 Mb and resulting in ~138-fold genome coverage. The PacBio SMRT sequencing data set consisted of 74,571 reads with an N50 read length of 17,770 bp and was used for de novo genome assembly. For the correction of indel errors, Illumina reads were mapped onto the newly assembled genome.

The genome of *E. coli* TolC (CP018801.1) consists of a single chromosome that is 4,792,200 bp long and contains 4,469 coding sequences (CDS), 88 tRNAs, 22 rRNAs, and 104 noncoding RNAs (ncRNAs). It was compared to all 259 fully sequenced *E. coli* genomes available from the National Center for Biotechnology Information (NCBI) via in silico DNA-DNA hybridization (isDDH), with isDDH values calculated by using the tool GGDC 2.1 (29). *E. coli* TolC showed the highest isDDH values (all isDDH values ≥98.28%) to *E. coli* strains K-12 MG1655 (NZ_CP014225.1), ER1821R (NZ_CP016018.1), NCM3722 (NZ_CP011495.1), K-12 W3110 (NC_007779.1), and JW5437-1 (NZ_CP014348.1).

A nucleotide-based genome BLAST distance phylogeny (GBDP) tree with branch support values inferred from both the nucleotide and amino acid data is depicted in Fig. S1 of our supplementary data posted on figshare (https://doi.org/10.6084/m9.figshare.5395471). The average branch support of the nucleotide tree was 47.3%, and branch support for the amino acid tree was 37.6%. Target strain *E. coli* TolC was placed in a highly supported subtree containing 14 strains, most of them K-12 strains.

Figure 1 shows the nucleotide sequence identity of *E. coli* TolC in comparison to the five most similar *E. coli* strains, as reported in BLAST+. Most notably, *E. coli* TolC contains the bacteriophage λ and the fertility plasmid F integrated into its chromosome. Phage λ was located between genes *ybhB* and *ybhC* at positions 3,079,545 to 3,128,200 of the *E. coli* TolC chromosome, and the F plasmid was integrated into an insertion segment element (IS3C) within the cryptic prophage DLP12 (positions...
3,368,702 to 3,467,447). This is in contrast to the most closely related E. coli strains, which encode neither the fertility plasmid nor phage /H9261, the only exception being NCM3722, which still carries phage /H9261 (Fig. 1A). In comparison to MG1655, an rph-1 mutation is absent in TolC, and the rpoS gene is present as the 33Am variant. Like other derivatives of E. coli K-12, strain E. coli TolC is also valine sensitive (ilvG deficient) (30).

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The tolC locus (btd92_00696) was inspected in detail, and the absence of a functional copy of the tolC gene was confirmed. The E. coli TolC strain carries a transposon insertion after base 1309 (c.1309_1310insIS5*) of the tolC gene, and this causes a disruption of the CDS (Fig. 1B). Genes of the three additional OMF proteins in E. coli (cusC, mdtQ, and mdtP) were not affected (see Table S1 at https://doi.org/10.6084/m9.figshare.5395471). The transposon within tolC was identified as transposable element IS5, which contains three protein-coding genes: the transposase gene insH1 (ins5A), insSB (**), and insSC (**) are indicated by arrows in reverse orientation, underneath insH1. A BLAST+ comparison of the tolC locus for each of the two strains indicated 100% nucleotide identity.

![FIG 1 Whole-genome comparison of E. coli TolC to closely related strains and a schematic presentation of transposon-mediated disruption of the tolC CDS in E. coli TolC. (A) BLAST ring image generator (BRIG) comparison of the E. coli TolC genome (innermost black ring) to the closely related genomes of E. coli strains K-12 MG1655, ER1821R, NCM3722, K-12 W3110, and JW5437-1 (the four outermost rings), shown in blue to red, respectively, as identified by isDDH (29). Shading of the four outermost rings is according to their respective percent nucleotide identity to the query sequence (E. coli TolC), determined by BLAST+. The second and third innermost rings show the GC skew (purple/green) and the GC content (black). IS5 elements are numbered according to annotations for E. coli K-12 MG1655 (NC_000913.3). The location of the fertility plasmid on the chromosome of E. coli TolC is indicated by the letter F (on left side of diagram). (B) Close-up comparison of the tolC locus of E. coli TolC and its closest relative, E. coli K-12 MG1655, drawn by using Easyfig (66). The tolC locus (tolC_1 and tolC_2) in E. coli TolC is interrupted by insertion of an IS5 element (IS5*) that codes for the transposase insH1 (ins5A). insSB (**), and insSC (**) are indicated by arrows in reverse orientation, underneath insH1. A BLAST+ comparison of the tolC locus for each of the two strains indicated 100% nucleotide identity.](https://doi.org/10.6084/m9.figshare.5395471)

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Genomic rearrangements of *E. coli* TolC in comparison with the most closely related strains. The complete genome of *E. coli* TolC was compared to the genomic sequences of *E. coli* K-12 MG1655 (A), *E. coli* ER1821R (B), *E. coli* NCM3722 (C), *E. coli* K-12 W3112 (D), and *E. coli* MG1655 JW5437-1 (E), and structural rearrangements were visualized using Easyfig (65). The relative locations of individual reference genes (in comparison to *E. coli* tolC in panel A) are indicated by gray arrows on the respective chromosomes (black horizontal lines). The nucleotide sequence identities, as determined using BLAST+, are indicated by different colored spectra: blue to red for translocations, and green to orange for inversions. Blue/green and red/orange indicate the highest (100%) and lowest (63%) detected sequence identities, respectively.
The strain described here was deposited at the Leibniz Institute DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and assigned strain number DSM 104619.

Role of TolC for MICs of carolacton and different classes of antibiotics. To evaluate the effect of TolC inactivation on antibiotic susceptibility of *E. coli*, the MICs of selected antibiotics against *E. coli* MG1655 and *E. coli* TolC were determined (Table 1). We included two RNA polymerase inhibitors, corallopyronin A and sorangicin, previously isolated from myxobacteria at our institution (34, 35).

*E. coli* TolC was at least 64 times more sensitive to carolacton than *E. coli* MG1655. The MIC of carolacton against *E. coli* TolC was in the same range as that reported by Jansen et al. (19). For *S. pneumoniae* TIGR4, the MIC of carolacton was determined to be 0.06 &micro;g/ml (24), similar to the value reported for *E. coli* TolC. In comparison to *E. coli* MG1655, *E. coli* TolC showed a strong increase in sensitivity (≥4-fold) to antibiotics from all functional groups. The determined MICs were in the same range as those reported previously for *E. coli* W3110 and its tolC null mutant (11), indicating that the presence of the F plasmid and phage λ do not affect antibiotic susceptibility. Rifampin and vancomycin are not substrates of the pump; thus, *E. coli* TolC is not expected to be hypersensitive to these compounds, which was confirmed. The data indicated that carolacton penetrates the two membranes of the Gram-negative cell envelope and that its intracellular inhibitory effect is comparable to that of Gram-positive cells.

### TABLE 1 MICs of antibiotics and carolacton against *E. coli* TolC and *E. coli* K-12 MG1655

| Mechanism and/or antibiotic | Target | MIC (µg/ml)* | E. coli K-12 MG1655 | E. coli TolC | FC* |
|-----------------------------|--------|--------------|---------------------|--------------|-----|
| Carolacton                  |        | ≥8           | 0.125               | 0.03         | 64  |
| Carolacton with (40 &micro;g/ml PAβN) |        | 4            | ≤0.03               | 128          |     |

**Protein biosynthesis**
- Chloramphenicol: 50S ribosomal subunit, MIC: 8 µg/ml, FC: 8
- Erythromycin: 50S ribosomal subunit, MIC: >64 µg/ml, FC: 32
- Gentamicin: 30S ribosomal subunit, MIC: 4 µg/ml, FC: 2
- Kanamycin: 30S ribosomal subunit, MIC: 8 µg/ml, FC: 4

**Peptidoglycan biosynthesis**
- Ampicillin: Penicillin-binding proteins, MIC: 16 µg/ml, FC: 4
- Cefotaxime: Penicillin-binding proteins, MIC: 0.0625 µg/ml, FC: 4
- Penicillin G: Penicillin-binding proteins, MIC: >32 µg/ml, FC: 16
- Vancomycin: d-Ala-d-Ala moieties of NAM/NAG peptides, MIC: >256 µg/ml, FC: 2
- Phosphomycin: UDP-N-acetylmuramoyl-N-acetylmuramic acid-3-enolpyruvyltransferase (MurA), MIC: ≥32 µg/ml, FC: 4

**Fatty acid biosynthesis**
- Triclosan: Enoyl-acyl carboxyl reductase (FabI), MIC: 0.125 µg/ml, FC: 16
- Cerulenin: β-keto-acyl-ACP synthase (FabB), MIC: ≥32 µg/ml, FC: 8

**RNA biosynthesis**
- Corallopyronin A: RNA polymerase, MIC: >32 µg/ml, FC: 16
- Rifampin: RNA polymerase, MIC: 16 µg/ml, FC: 8
- Sorangicin: RNA polymerase, MIC: 16 µg/ml, FC: 1

**Cell division**
- Novobiocin: DNA gyrase, MIC: >16 µg/ml, FC: 16
- Ciprofloxacin: DNA gyrase, MIC: 0.015 µg/ml, FC: 4

**Folate biosynthesis**
- Trimethoprim: Dihydrofolate reductase (FolA), MIC: 0.5 µg/ml, FC: 8
- Sulfamethoxazole: Dihydropteroate synthase (FolP), MIC: 128 µg/ml, FC: 64

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*Boldface values indicate that MICs for the MG1655 control strain differed by ≥4-fold. The FC increase in susceptibility of *E. coli* TolC relative to *E. coli* MG1655 susceptibility. NAM, N-acetylmuramic acid; NAG, N-acetylglucosamine.
Transcriptional response of *E. coli* TolC to carolacton. We analyzed the transcriptome of carolacton-treated cultures of *E. coli* TolC in comparison to untreated cultures during the first 30 min of growth. In total, 4,730 transcripts of *E. coli* TolC were investigated using Rockhopper (see Data Set S1 in the supplemental material). At 30 min after addition of carolacton, 71 transcripts showed a strong differential abundance (log2 fold change [FC] of ≥±2), corresponding to 1.6% of all open reading frames of *E. coli* TolC (Data Set S2). At this time point, *E. coli* TolC grows at the same rate with or without carolacton (see below). The data therefore provide additional proof that carolacton immediately enters the Gram-negative cell. At a log2 FC of ≥±0.8, approximately 29% of all genes were differentially abundant, comparable to the degree of differential transcript abundance in *S. mutans* (31.3%) and *S. pneumoniae* (22.8%) in the presence of carolacton when we used an identical cutoff (21, 24). The most strongly differentially abundant transcripts encoded components for flagellar assembly, heat shock and cold shock proteins, and chaperones (Fig. 3). Transcription of the alternative sigma factor F (σ28) was upregulated ~7.4-fold (log2, FC, 2.88), and the putative helix-turn-helix (HTH)-type transcriptional regulator RhmR was downregulated. Moreover, precursors of the outer membrane pore proteins NmpC (btd92_03329) and PhoE (btd92_03746) were upregulated. Interestingly, all 7 StyR-44 family small noncoding RNAs encoded in the genome were strongly (log2 FC, ≥6.5) upregulated after only 5 min of growth with carolacton. The data showed that interaction of *E. coli* TolC with carolacton triggers global transcriptional adaptations already after 5 min, suggesting a molecular target in a central metabolic pathway.  

Stereospecificity of carolacton activity and inhibition of efflux. Subsequently, the differences in carolacton susceptibility between *E. coli* TolC and *E. coli* MG1655 were investigated in detail over all growth phases. *E. coli* MG1655 with and without carolacton and TolC without carolacton grew similarly and reached their maximal optical density at 600 nm (OD600) of ~6 after 7 h (Fig. 4A). In the presence of carolacton (added at t = 0), growth was indistinguishable from the controls for 1 h. At this time point, growth of the carolacton-treated culture of the *E. coli* TolC strain was strongly inhibited, while all other strains entered the exponential growth phase. The carolacton-treated culture of the *E. coli* TolC strain grew linearly over the next 5 h to an OD600 of approximately 0.8, which did not increase much farther and reached a maximal OD600 of around 1 after 24 h. Complementation of *E. coli* TolC with a plasmid-borne copy of the OMF TolC was able to restore insensitivity to carolacton, confirming indeed the absence of TolC-mediated efflux of carolacton as the sole cause for sensitivity (Fig. 5).  
epi-Carolacton is a carolacton epimer with an inversion of the stereocenter at C-9 from the native (S) to the (R) configuration. This carolacton derivative lacks biological activity in *S. pneumoniae* TIGR4 and *S. mutans* UA159 (22, 24). Here, we tested the inhibitory properties of epi-carolacton against *E. coli* TolC. Figure 4B shows that epi-carolacton had no influence on growth of *E. coli* TolC. Since epi-carolacton was dissolved in dimethyl sulfoxide (DMSO), we investigated its effect on growth as an additional control, but we did not detect any. The loss of growth inhibition of epi-carolacton shown here suggests that the molecular target of carolacton might not only be conserved in the genus *Streptococcus* but also in the phyla *Firmicutes* and *Proteobacteria.*  

Antibiotics that are substrates of TolC have to be administered in high doses to overcome the intrinsic resistance mediated by efflux (13). Alternatively, they could be applied in combination with efflux pump inhibitors. Therefore, we investigated the influence of PAβN, a competitive inhibitor of AcrAB-TolC (16), on carolacton sensitivity in *E. coli*. Table 1 shows that the MIC of *E. coli* MG1655 toward carolacton was reduced from >8 μg/ml to 4 μg/ml when PAβN was coadministered at 40 μg/ml. Lower concentrations of PAβN had no effect on the MIC of carolacton. The susceptibility of the TolC mutant was also increased by PAβN. The MIC of *E. coli* against PAβN has been shown before to be strongly reduced in an efflux-deficient strain (ΔacrAB); moreover, PAβN can cause membrane destabilization as an unspecific side
Accordingly, we observed a growth reduction of ~45% for the efflux-deficient E. coli TolC strain when grown with 40 µg/ml PA/H9252/H11350/H11006/H11349/H0.01, for at least one sample during the time course.

Finally, we investigated the role of PA/H9252/H11350/H11006/H11349/H0.01 under the same conditions as those used for studying the effect of TolC deletion. The effect of PA/H9252/H11350/H11006/H11349/H0.01 on growth inhibition of E. coli MG1655 by carolacton was dependent on the concentration of PA/H9252/H11350/H11006/H11349/H0.01. At concentrations of 20 and 40 µg/ml PA/H9252/H11350/H11006/H11349/H0.01, a maximal inhibition of 59% and 78%, respectively, was found, in comparison to a culture treated with only carolacton. The observations concerning MICs and a PA/H9252/H11350/H11006/H11349/H0.01-mediated growth inhibition by carolacton were reproducible for the tolC-complemented E. coli TolC strain.

FIG 3  The most strongly differentially abundant transcripts in E. coli TolC during growth with carolacton (0.25 µg/ml). (A) Overview; (B) the most strongly differently regulated ncRNAs. The cutoff for differentially abundant transcripts was set at log2 FC of ≥ 2 for general transcripts and ≥ 2.5 for ncRNAs (FDR, ≤0.01), for at least one sample during the time course.
For comparison, inhibition of growth of *E. coli* TolC treated with carolacton is shown, which reached a maximum of 90% in comparison to the untreated culture (Fig. 6B). Thus, in *E. coli*, addition of 40 µg/ml PA/H9252, together with carolacton, causes a growth reduction similar to that with treatment with carolacton in a TolC-deficient strain.

The observed growth inhibition characteristics of carolacton- and PAβN-treated cultures of *E. coli* TolC and *E. coli* MG1655 were also reflected in drastic changes in the maximal doubling time (*t_D*) of cells during exponential growth (Table 3). The *t_D* of *E. coli* TolC after treatment with carolacton increased from 25 to >372 min (Fig. 4 and 7). A comparable decrease of the doubling time was also observed after coadministration of PAβN and carolacton to cultures of *E. coli* MG1655 (*t_D* ~257 min), supporting the
previous observation that PAβN treatment can facilitate a carolacton-dependent slowdown of cell division and consequently growth inhibition of an otherwise-resistant strain.

**DISCUSSION**

Here we studied the role of TolC, a component of the major multidrug efflux system of *E. coli*, in its susceptibility to carolacton. To this end, we determined the genome...
sequence of the genetically uncharacterized, highly carolacton-susceptible E. coli TolC strain and revealed that it (i) shares the highest nucleotide sequence homology with E. coli MG1655 and (ii) is also phylogenetically reliably placed in a highly supported group that primarily harbors other K-12 strains. Originally, in the 1950s, the chromosome of the wild-type E. coli K-12 was cured from phage λ, generating E. coli K-12 W1485. E. coli K-12 W1485 was subsequently cured of its F+ factor to make MG1655 (36). Thus, as E. coli TolC still contains the phage λ and a chromosomal copy of the F plasmid, our TolC strain appears to be an ancient prototrophic derivative of the original wild-type E. coli K-12. The profile of MIC resistance of E. coli TolC provided further evidence for an impairment of the efflux function in the mutant strain, rather than a change in the permeability of the outer membrane (25). As the biological function of the TolC OMP is of great scientific interest, tolC deletion mutants of E. coli are often generated anew, elaborately and with varied techniques for every study and in different, often-undescribed genetic backgrounds (25, 27, 28). The E. coli TolC strain sequenced here has now been thoroughly characterized. It is closely related to the ancestral E. coli wild-type strain K-12 and publicly available and thus could be used as a standard tool in the future.

A strong growth inhibition of E. coli TolC occurred at 0.25 &micro;g/ml (0.54 &micro;M). At this concentration, growth of S. pneumoniae TIGR4 is inhibited in a similar way, indicating a bacteriostatic role of carolacton (24). The same concentration of carolacton caused cell death in biofilms of S. mutans (20). A carolacton epimer, C-9 (R) (epi-carolacton), lacked biological activity in all organisms tested so far (22, 24). Here, we showed that it was also inactive when testing growth of the highly carolacton-sensitive E. coli TolC strain. The complete loss of biological activity of this carolacton derivative, with a mere inversion of a single stereogenic center at C-9, indicates a specific interaction of carolacton with a cellular target. A target that is present not only in streptococci (24) but also in Gram-negative bacteria like Aggregatibacter (22) and E. coli, and thus might be conserved in the phyla Firmicutes and Proteobacteria.

The data demonstrate that carolacton can enter the Gram-negative cell but is a substrate of the tripartite multidrug efflux pump AcrAB-TolC, the main component of intrinsic antibiotic resistance in Enterobacteriaceae. Its clinical application would therefore require high concentrations, or could be combined with efflux pump inhibitors. Treatment of the E. coli MG1655 with 40 &micro;g/ml of PAβN, specific for inhibition of the AcrAB-TolC and AcrEF-TolC efflux complexes (16), rendered the strain susceptible to

### TABLE 2

| Strain                      | MIC of carolacton (µg/ml) |
|----------------------------|---------------------------|
| E. coli TolC/pIB166-tolC   | >8                        |
| E. coli TolC/pIB166-tolC (with 40 µg/ml PAβN) | 2                      |

### TABLE 3

Effect of carolacton treatment and PAβN on growth kinetics of the E. coli strains

| E. coli strain and treatment | Maximum specific growth rate (µmolar t<sup>max</sup>) | Doubling time (min) |
|-----------------------------|------------------------------------------------------|---------------------|
|                             | Control | Carolacton | Control | Carolacton |
| MG1655                      | 1.65 (±0.03) | 1.67 (±0.05) | 25.1 (±0.4) | 25.0 (±0.7) |
| MG1655, 40 &µg/ml PAβN      | 0.77 (±0.05) | **0.17 (±0.05)** | **54.3 (±3.6)** | **257.2 (±59)** |
| TolC                        | 1.66 (±0.05) | **0.11 (±10<sup>-3</sup>)** | **25.0 (±0.7)** | **372.5 (±6)** |
| TolC, 40 &µg/ml PAβN        | 0.82 (±0.05) | **0.05 (±10<sup>-3</sup>)** | **51.0 (±2.9)** | **899.5 (±44)** |
| TolC, epi-carolacton        | 1.68 (±0.01) | 1.67 (±0.02) | 24.7 (±0.2) | 24.9 (±0.3) |
| TolC/pIB166-tolC            | 1.63 (±0.19) | 1.54 (±0.2) | 25.9 (±3.1) | 27.4 (±3.5) |
| TolC/pIB166-tolC, 40 &µg/ml PAβN | 0.75 (±0.04) | **0.11 (±10<sup>-3</sup>)** | 55.4 (±0.6) | 451.9 (±190) |

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*Carolacton was applied at a final concentration of 0.25 &micro;g/ml, except when epi-carolacton was used for treatment at the same final concentration.

*Values in boldface were significantly different from the control, based on a two-tailed Student’s t test. **, P < 0.001; *, P < 0.01.
carolacton in a similar way as the deletion of TolC. The effect of AcrEF for the export of carolacton can be neglected here, as its expression is very low and this exporter has a primary role in cell division (37); hence, deletion of acrEF does not change the antibiotic resistance phenotype of *E. coli* (11). Interestingly, lower concentrations of PAβN did not influence the sensitivity to carolacton at all, which is puzzling, because carolacton was provided at 0.25 µg/ml and inhibition by PAβN has been reported to be competitive (16).

The RNA-seq data for *E. coli* TolC indicated a strong regulatory response upon treatment with carolacton within the first 30 min, where growth is still unaffected, confirming the entry of carolacton into the cell and its likely immediate interaction with an intracellular target. The observed changes involved small regulatory RNAs, a sigma factor, chaperones, heat and cold shock proteins, flagellar components, and membrane transport proteins. The sigma factor F (σ28 in *E. coli*) is needed for flagellar assembly and motility (38), in accordance with the upregulation of the flagellar components *fliL* (*btd92_01821*) or *fliJ* (*btd92_01823*). Interestingly, all ncRNAs of the StyR-44 family were strongly upregulated already at the 5-min time point. StyR-44 ncRNAs are found in ribosomal operons located upstream of the 23S rRNA; their expression is dependent on the growth rate, but their specific function is unknown (39). As ncRNAs are known to act as global regulators of gene expression (40), their differential transcript abundance shows a fast and strong global regulatory response to carolacton. Carolacton treatment also caused upregulation of the outer membrane pore proteins NmpC (Log2, FC, 2.72) and PhoE (Log2, FC, 2.85), both of which play a role under heat shock and phosphorus starvation conditions, respectively (41, 42). The transcriptome data showed that the molecular target of carolacton may be located within a central metabolic pathway in the cell, and inhibition of this target induces multiple metabolic and transcriptional adaptations.

In conclusion, we found that carolacton efficiently penetrates the Gram-negative cell envelope, and low micromolar concentrations are sufficient for growth inhibition of *E. coli*, unless it is exported by the tripartite AcrAB-TolC efflux system. Carolacton might potentially be used against Gram-negative bacteria in combination with EPIs.
**TABLE 4** *E. coli* strains and plasmids used in this study.

| Strain or plasmid | Relevant genotype or description | Reference or source |
|-------------------|---------------------------------|---------------------|
| **Strains**       |                                 |                     |
| DH5α              | Cloning strain                  | Stratagene          |
| K-12 MG1655       | F^- λ^- ΔlvG rfb-50 rph-1       | DSM 18039           |
| TolC              | F^+ λ^+ ΔlvG rfb-50 ropS(33Am) ΔtolC | Laboratory collection, DSM 104619 |
| TolC/pIB166-tolC  | TolC strain containing pIB166-tolC for complementation of strain TolC, Cm' | This work |
| **Plasmids**      |                                 |                     |
| pIB166            | Cm' Removal of P23 and integration of tolC under control of its native promoter (P_{tolC-tolC}), Cm' | 69 This work |
| pIB166-tolC       |                                 |                     |

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *E. coli* strains used for growth experiments (Table 4) were routinely grown under aerobic conditions in Luria-Bertani (LB) broth overnight (o/n) at 37°C (200 rpm). The cultures were then used to inoculate fresh LB medium to an OD_600 of 0.05, which was determined photospectrometrically (Ultrospec 3100 Pro; Amersham Biosciences, Inc.). Cultures with an OD_600 of >0.5 were diluted in LB broth to below 0.5 in order to maintain the linearity between the measured absorbance and cell density and to achieve the most exact results. The initial culture was then split into equal volumes and supplemented with carolacton, 9(R)-epi-carolacton, or PAβN, or maintained as untreated controls. For cryo-conservation, *E. coli* was grown in LB o/n, mixed with an equal volume of 50% (vol/vol) glycerol in cryovials, and frozen at −80°C.

**Storage of carolacton, epi-carolacton, and PAβN.** Carolacton and its derivative 9(R)-carolacton were dissolved in methanol or DMSO to a final concentration of 5.3 mM (250 µg/ml) or 2 mM (94.3 µg/ml), respectively, and stored in small aliquots in amber glass vials at −20°C in the dark. PAβN (25 mg/ml in H_2O) was stored at −20°C and used at final concentrations between 5 and 40 µg/ml, as indicated.

**Complementation of *E. coli* TolC.** Chemo-competent cells of *E. coli* were prepared according to the TSS method described by Chung et al. (43). pIB166 was PCR amplified with Phusion polymerase (NEB) using primers (pIB166_fwd and pIB166_rev), thereby eliminating P23 (Table 5). Genomic DNA of *E. coli* K-12 MG1655 served as a template for PCR amplification of the tolC locus (b3035), using primers (tolC_fwd and tolC_rev), additionally introducing flanks homologous to the linearized vector sequence. PCR products were purified with a PCR purification kit (Qiagen, Germany). The PCR-amplified tolC gene was cloned into pIB166 by using the CloneEZ kit (Genescript), and the reaction mix was transformed into *E. coli* DH5α. Obtained plasmids were verified by sequencing and subsequently transformed into *E. coli* TolC. *E. coli* transformed with pIB166 or its derivatives were grown on LB agar plates or in liquid LB broth containing 20 µg/ml chloramphenicol.

**Determination of MIC values.** MIC values of selected antibiotics and of carolacton against *E. coli* and *E. coli* K-12 MG1655 were determined by 2-fold serial microdilution in LB broth with incubation at 37°C for 20 h, as described previously (44). Antibiotics were tested in the following dilution ranges: ampicillin (32 to 0.25 µg/ml), carolacton (8 to 0.03 µg/ml), cepoxaxime (1 to 0.078 µg/ml), cefuroxime (32 to 0.25 µg/ml), ciprofloxacin (0.25 to 0.0019 µg/ml), chloramphenicol (64 to 0.5 µg/ml), gentamicin (32 to 0.25 µg/ml), kanamycin (8 to 0.03 µg/ml), novobiocin (16 to 0.125 µg/ml), penicillin G (32 to 0.25 µg/ml), phosphomycin (32 to 0.25 µg/ml), rifampin (32 to 0.25 µg/ml), sorafolin (32 to 0.25 µg/ml), sulfamethoxazole (256 to 2 µg/ml), trimethoprim (2 to 0.015 µg/ml), and vancomycin (256 to 2 µg/ml), if not indicated otherwise. Corallopyronin A and sorafolin were kindly provided by Rolf Jansen (HZI, Braunschweig). Antibiotics were purchased from Sigma-Aldrich (Steinheim, Germany) or Carl Roth GmbH (Karlsruhe, Germany). MICs were the lowest concentrations that did not yield visible bacterial growth. The cell count of the initial inoculum was 5×10^9 CFU/ml, which was confirmed by plating of serial cell dilutions and counting of CFU. MICs were confirmed in at least two independent experiments.

**TABLE 5** Overview of oligonucleotides used

| Primer | Sequence (5’–3’) | Purpose | Reference |
|--------|------------------|---------|-----------|
| pIB166_fwd | AATTCTAGACCTCGAGATCTATCGATAAGC | Linearization of pIB166 | This work |
| pIB166_fwd | CAGCTTCTGATGTATTTTTATATTTAC | | |
| tolC_fwd | ATCACGAGCTAAAGGCAGTCGCGGAGGAGATGAACTAATGGTAAATGGTGAATTTCC | Cloning of tolC (b3035) | This work |
| tolC_rev | CGTCGACGTTTAATCGGACTAGGCAGACGAAGGGTTATGAGCGTTACTCGGTTG | | |
Growth kinetics. The maximal specific growth rate ($\mu_{\text{max}}$, per hour) and doubling time ($t_D$, in minutes) of bacteria were determined from semilogarithmically transformed growth curves (Fig. 8) according to methods described previously (45).

Extraction of genomic DNA and PacBio/Illumina sequencing. Genomic DNA of *E. coli* TolC was extracted by gravity flow using the Genomic-tip 20/G kit (Qiagen, Germany). Purified genomic DNA of *E. coli* TolC was processed for PacBio SMRT sequencing and Illumina MiSeq paired-end sequencing (2 × 250 bp) with a target genome coverage of 150-fold. DNA libraries for MiSeq sequencing of the genome of *E. coli* TolC were prepared with the NEBNext Ultra DNA library prep kit for Illumina sequencing (New England Biolabs, Ipswich, MA). Quality controls of NEBNext Ultra DNA libraries were conducted by fluorometric quantitation using the Qubit 3.0 fluorometer (Thermo, Fisher Scientific, Germany). For PacBio SMRT sequencing, a PacBio SMRTbell library was constructed according to the manufacturer’s instructions and the library was sequenced on the PacBio RSII platform. De novo genome assemblies were built with PacBio’s SMRT Portal (v.2.3.0) by utilizing the Hierarchical Genome Assembly Process 3 (HGAP3) (46). The genome was error corrected against indel errors by a mapping of Illumina reads onto finished genomes, using BWA (47) with subsequent variant and consensus calling using VarScan (48); automated sequence annotation was performed with Prokka (v.1.8) (49).

RNA isolation. Overnight cultures of *E. coli* TolC (OD<sub>600</sub>, ~5) were diluted 1:200 in LB broth and grown to an OD<sub>600</sub> of 0.1. The culture was subsequently divided into equal parts: one part was treated with 0.25 µg/ml carolacton, and the other part was treated with an equal volume of solvent (methanol). Cells were sampled before treatment and at 5, 15, and 30 min post-addition of carolacton. The samples were transferred to an equal volume of RNAProtect (Qiagen, Germany) and incubated for 5 min at room temperature. Cells were pelleted (13,000 rpm, 2 min), the supernatant was removed, and the pellet was frozen at −80°C. For RNA extraction, the pellets were washed with 0.5 ml nuclease-free water and centrifuged (13,000 rpm, 2 min). RNA extraction was carried out using the miRNeasy minikit (Qiagen, Germany) according to the manufacturer’s instructions for purification of total RNA. The removal of genomic DNA was carried out by the optional on-column DNase I digestion using the DNase I kit (Qiagen, Germany) for 45 min. After the washing steps, the RNA was eluted in 50 &mu;g/ml of nuclease-free water supplied with the kit. To test the integrity of the isolated total RNA and the enriched mRNA, samples were analyzed using the Agilent 2100 Bioanalyzer and the RNA 6000 Pico kit (Agilent, Germany).

Enrichment of mRNA and RNA sequencing. mRNA enrichment was achieved by using the RiboZero kit for Gram-negative bacteria (epicenter; Illumina) for 2 &mu;g of total RNA as described by the manufacturer. Successful removal of rRNA was verified using an Agilent 2100 Bioanalyzer (Agilent, Germany). Direct strand-specific RNA sequencing was performed using the Illumina HiSeq 2500 platform (Illumina) according to the ScriptSeq v.2 protocol for RNA-seq library construction (Agilent, Germany). After quality control and clipping of adapter sequences (primers and bar codes), mapping of reads and data analysis was conducted using the Rockhopper software (v.2.0.3) (50).

RNA-seq data analysis. Trimming of Illumina sequencing adapter sequences of obtained reads was achieved using fastq-mcf (51). Reads were mapped to the *E. coli* TolC genome (CP018801.1), and the read counts per feature were determined with Rockhopper (v.2.0.3) (50, 52). For analysis of differential abundance of transcripts, the raw read counts obtained with Rockhopper (53) were used, and changes in transcript abundance levels were calculated with the Bioconductor edgeR package (v.3.1) for R.
Whole-genome-based phylogenomic analyses. To elucidate the phylogenetic positioning of strain TolC, and given its high sequence similarity to strain E. coli K-12 MG1655, a member of phylogroup A (58), a corresponding reference data set was defined. The latter included all 32 members of phylogroup A, according to methods described previously (58), and was further complemented by four recently genome-sequenced strains that had been found to be highly similar to TolC (accession numbers NZ_CP011495, NZ_CP014225, NZ_CP014348, and NZ_CP016018). Two whole-genome-based phylogenomic analyses were conducted using the genome BLAST distance phylogeny approach (59) in its latest version (29). The first analysis was based on the nucleotide data restricted to genes, whereas the second one used protein data only. Coding regions were determined via Prodigal under default settings (54). False-discovery rate (FDR)-adjusted P values were calculated according to methods described previously (56). FDR values of < 0.01 were considered significant. Heat maps were generated for genes that showed a log FC of ≥ 2 for at least one time point (FDR, < 0.01), log FC values of transcript abundance obtained with edgeR were used as input for the heatmap2 function of the R package gplots (v.2.15.0) (57).

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/mSphereDirect.00375-17.
DATA SET S1, XLSX file, 0.5 MB.
DATA SET S2, XLSX file, 0.02 MB.

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