Transcriptional Analysis of Long-Term Adaptation of *Yersinia enterocolitica* to Low-Temperature Growth

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To analyze the transcriptional response of *Yersinia enterocolitica* cells to prolonged growth at low temperature, a collection of *luxCDABE* transposon mutants was cultivated in parallel at optimal (30°C) and suboptimal (10°C) temperatures and screened for enhanced promoter activities during growth until entering stationary phase. Among 5,700 *Y. enterocolitica* mutants, 42 transcriptional units were identified with strongly enhanced or reduced promoter activity at 10°C compared to 30°C, and changes in their transcriptional levels over time were measured. Green fluorescent protein fusions to 10 promoter regions confirmed the data. The temporal order of induction of the temperature-responsive genes of *Y. enterocolitica* was deduced, starting with the expression of cold shock genes *cspA* and *cspB* and the elevated transcription of a glutamate-aspartate symporter. Subsequently, cold-adapted cells drastically up-regulated genes encoding environmental sensors and regulators, such as UhpABC, ArcA, and methyl-accepting chemotaxis protein I (MCPI). Among the most prominent cold-responsive elements that were transcriptionally induced during growth in early and middle exponential phase are the insecticidal toxin genes *tcaA* and *tcaB*, as well as genes involved in flagellar synthesis and chemotaxis. The expression pattern of the late-exponential- to early-stationary-growth phase is dominated by factors involved in biodegradative metabolism, namely, a histidine ammonia lyase, three enzymes responsible for uptake and utilization of glycogen, the urease complex, and a subtilisin-like protease. Double-knockout mutants and complementation studies demonstrate inhibitory effects of MCPI and UhpC on the expression of a putative hemolysin transporter. The data partially delineate the spectrum of gene expression of *Y. enterocolitica* at environmental temperatures, providing evidence that an as-yet-unknown insect phase is part of the life cycle of this human pathogen.

A hallmark of microorganisms is their adaptability to environmental changes. Many bacteria are likely to encounter several alterations in milieu during their life cycle and must therefore be able to sense and adequately respond to these changes (1). Major contributions to our understanding of the complex molecular mechanisms underlying the successful adaptation come from the study of pathogenic bacteria. Pathogens such as *Salmonella enterica* and *Listeria monocytogenes* have temperature-regulated genes, obviously because their life cycles involve growth in a mammalian host, as well as exposure to environmental temperatures. An association between temperature and gene regulation has long been established in the yersiniae, due to their biphasic life cycles, during which they encounter a variety of ambient conditions (31, 64). *Yersinia pestis*, the causative agent of plague, regulates genes encoding capsule, iron transporters, outer membrane proteins, and toxins in response to temperatures found in the flea vector or in the mammalian host (37). In *Yersinia enterocolitica*, the Yop proteins and YadA are only produced at 37°C (14), while the invasin gene *inv* is maximally expressed at 26°C and is repressed at body temperature (20). To identify genes playing a role in host temperature adaptation, DNA microarray analysis was applied to, e.g., group A *Streptococcus* (61), *Borelia burgdorferi* (51, 62), *Campylobacter jejuni* (63), and *Y. pestis* (27).

Considerable knowledge about the response to a temperature downshift comes from studies of mesophilic microorganisms such as *Escherichia coli* (34) and *Bacillus subtilis* (25, 73), as well as of psychrotolerant microorganisms such as *L. monocytogenes* (5), *Arthrobacter globiformis* (7), *Pseudomonas fragi* (29), and *Y. enterocolitica* (45). Extended by the application of macro- and microarray techniques (6, 35, 54), these data revealed specific mechanisms necessary for cold shock adaptation (48). Those requirements include modifications of the translation apparatus and the membrane that occur within a period of approximately 3 h after a sudden temperature drop (52, 73). This acclimatization phase is characterized by an increased but transient expression of cold shock-induced proteins like CspA, NusA, GyrA, RecA, IF-2, or polynucleotide phosphorylase and by cold acclimatization proteins continuously expressed at elevated levels (26, 30, 48, 66). By selective capture of transcribed sequences, it was shown only recently that *L. monocytogenes* acclimatization to 10°C probably involves general microbial stress responses and alterations in amino acid metabolism, as well as degradative metabolism (39).

However, most studies of differential gene expression in response to alterations in environmental conditions have been restricted to few target loci or, applying microarray technologies, to single time points, but no systematic, real-time analyses of gene expression of psychrotolerant bacteria following cold acclimatization have been available so far. Underused reporter gene technologies based on bioluminescence are able to overcome such limitations (55, 69). The use of promoter fusions with bioluminescent *luxCDABE* genes provides an independent analysis method compared to hybridization experiments.

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as demonstrated by the investigation of E. coli gene responses to DNA damage (69). Since the luxCDABE system produces light without the addition of exogenous substrate, it allows the efficient, facile, and repeatable measurement of promoter activities at many time points. A further advantage of the luciferase system over the green fluorescent protein (GFP) reporter is its high sensitivity, which enables the detection of transcriptional signals over a large dynamic range and thus the identification of down-regulated genes (68).

To describe the long-term response of a pathogen to low temperature, we chose Y. enterocolitica, a bacterium that is widely distributed in nature in aquatic and terrestrial reservoirs, as well as in animals. The presence of this bacterium in frozen and chilled food products is of major concern, since it may give rise to severe gastrointestinal illness (8, 9). Genome-wide transposon mutagenesis of strain W22703 was carried out using a plasmid-borne mini-Tn-based promoter

<table>
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<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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TABLE 1. Strains and plasmids used in this study

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. All cultures were grown in Luria-Bertani (LB) broth (10-g/liter tryptone, 5-g/liter yeast extract, 5-g/liter NaCl), or on LB agar (LB broth supplemented with 1.5% agar). E. coli was grown at 37°C, and Y. enterocolitica was grown at the indicated temperature. If appropriate, the media were supplemented with the following antibiotics: kanamycin (50 μg/ml), nalidixic acid (20 μg/ml), tetracycline (12 μg/ml), or chloramphenicol (20 μg/ml). Strains were frozen in 13% glycerol at -70°C for permanent collection.
luxCDABE reporter mutant library. Transposon-bearing suicide plasmid pUT mini-Tn5 luxCDABE Km2 (17, 74) was transferred from E. coli donor strain S17.1 hpr (60) to acceptor strain Y. enterocolitica W22703 (14) by liquid mating as previously described (74) with the following modifications: donor and acceptor strains were grown to an optical density at 600 nm (OD600) of 0.5, and 100 µl of each culture was mixed and supplied with 800 µl of 10 mM MgSO4 solution. Following an incubation time of 5 min at room temperature (RT), cells were pelleted (3,000 rpm; 5 min at RT), resuspended in 100 µl LB broth, and spread onto antibiotic-free LB agar plates. The mating was incubated for 24 h at 30°C, and approximately 20% of the bacterial lawn was scraped off carefully and resuspended in 1 ml LB broth containing 13% glycerol. To avoid redundancy of the mutant library, five independent matings were performed, and aliquots of each mating were frozen in liquid N2 and stored at −70°C. Frozen aliquots were adjusted with LB medium to an OD600 of ~0.3. Subsequently, 100 µl of this suspension was plated on LB selection plates (145-mm diameter) with kanamycin (200 µg/ml) and nalidixic acid and incubated for 24 h at 30°C. From each mating, 2,400 to 3,600 mutants were obtained, and each third colony was individually transferred to 96-well master plates filled with 800 µl LB containing antibiotics, as above, per well. Cells were cultivated for 16 h at 30°C on a shaker for microtiter plates. These overnight cultures were used for a subsequent luciferase-screening above, per well. Cells were cultivated for 16 h at 30°C on a shaker for microtiter plates. The illustration depicts the transcriptional fusion of luxCDABE to the cspB promoter as a result of transposon mutagenesis. Position and orientation of the genes are indicated by arrows, and the mini-Tn5 transposon flanked by the O and I end is marked by dotted regions. Km′, kanamycin resistance gene; bla, beta-lactamase gene.

FIG. 1. Example of gene arrangement after insertion of the promoterless luxCDABE operon into Y. enterocolitica cspB. The suicide plasmid pUT mini-Tn5 luxCDABE Km2 was transferred via conjugation into Y. enterocolitica W22703, followed by random insertion of the promoterless luxCDABE operon and a kanamycin resistance gene into the Y. enterocolitica genome. The illustration depicts the transcriptional fusion of luxCDABE to the cspB promoter as a result of transposon mutagenesis. Position and orientation of the genes are indicated by arrows, and the mini-Tn5 transposon flanked by the O and I end is marked by dotted regions. Km′, kanamycin resistance gene; bla, beta-lactamase gene.
end or the I-end. Each obtained sequence was screened for ClaI, HindIII, SphI, SspI, or DraI restriction sites and for the partial transposon sequence. Mapping of the mini-Tn5 luxCDABE insertions was conducted by using the Y. enterocolitica BLAST Server from the Sanger Institute (http://www.sanger.ac.uk/Projects/Y_enterocolitica/). Sequences without similarities to sequenced strain Y. enterocolitica 8081 were classified as specific for strain W22703. Promoter sequences located upstream of the transposon insertions were identified using a promoter prediction program (http://www.fruitfly.org/seq_tools/promoter.html).

TABLE 2. Primers used in this study

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"I-end sequencing."  
"O-end sequencing."  
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"Recognition sites of restriction enzymes are underlined."
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<td>tca&lt;sup&gt;c&lt;/sup&gt;A</td>
<td>Putative toxin subunit</td>
<td>P-tca&lt;sup&gt;c&lt;/sup&gt;A-luxCDABE</td>
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<td>Virulence</td>
<td>12</td>
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<td>Urea operon</td>
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<td>Alkaline serine protease (subtilisin)</td>
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<td>YE4013</td>
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<td>P-luxCDABE-YE4013&lt;sup&gt;c&lt;/sup&gt;-YE4012&lt;sup&gt;c&lt;/sup&gt;-YE4011</td>
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<td>Histidine ammonia-lyase (histidase), HatH</td>
<td>P-YE3021&lt;sup&gt;c&lt;/sup&gt;-luxCDABE-YE3020</td>
<td>0.89</td>
<td>Metabolism</td>
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<td>luxCDABE</td>
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</table>

Continued on following page
promoter regions were amplified from purified /H9251 MCR. Plasmids containing the correct transcriptional
Hudson, N.H.) into the SalI/EcoRI site of pPROBE-NT (42) and transformed
and SalI (MBI Fermentas, Vilnius, Lithunia), ligated (T4 DNA ligase; Gibco,
PCR using the primers listed in Table 2. PCR products were digested with EcoRI
bearing the recombinant
Taufkirchen, Germany) and transformed into
and W22703-YE0480::
Y. enterocolitica
were amplified from
Y. enterocolitica
strain by plate mating (see above). Transformants were selected on
chromosomal DNA using the primers listed

Y. enterocolitica
transposon mutants with and without the insertional
knots were grown in parallel at 30°C and 10°C to measure bioluminescence
Minim-Tn5 luxCDABE genes were inserted in opposite to the putative promoters of ymoA and YE0951.
Transposon insertion was in strain NCTC10460.

As shown by a gfp fusion construct, the luxCDABE insertion is transcribed from the promoter upstream of ureD.

Construction of insertional knockout mutants and complementing vectors. To
generate knockout mutants of uhpC, yahM, and YE2575 by plasmid insertion via
homologous recombination, short intragenic fragments from the target genes
were amplified from Y. enterocolitica chromosomal DNA using the primers listed in
Table 2. Fragments were digested with XbaI (MBI Fermentas, Vilnius, Lithuania) and SacI (New England Biolabs, Beverly, Mass.) and ligated into XbaI/SacI-
restricted suicide plasmid pKRG9. The recombinant plasmids were transformed
into E. coli S17.1 spr by electroporation and transferred into each individual Y. enterocolitica strain by plate mating (see above). Transformants were selected on pla-
tes carrying naldixic acid, kanamycin, and chloramphenicol. To exclude
 illegitimate recombination, the correct insertion of the recombinant plasmid was confirmed by PCR using a gene-specific test primer and a plasmid-derived primer. Y. enterocolitica transposon mutants with and without the insertional
knots were grown in parallel at 30°C and 10°C to measure bioluminescence and
optical density as described above. To complement the double mutants W22703-YE0480::lux-YE2575 and W22703-YE0480::lux-uhpC, the coding
sequences of YE2575 and uhpC, the first gene together with 362 bp of its upstream
sequence, were amplified with the appropriate oligonucleotides listed in Table 2.
The resulting fragments were cloned via ScaI (YE2575) and EcoRI (uhpC) into
pACYC184. In the resulting recombinant plasmids, pACYC-YE2575 and
pACYC-uhpC, the direction of transcription of both genes corresponds to that of
the disrupted gene encoding chloramphenicol acetyltransferase. The constructs
were verified by PCR and restriction analysis and transformed by electroporation
into W22703-YE0480::lux-YE2575 and W22703-YE0480::lux-uhpC, respectively.

Construction of transcriptional gfp fusions and in vitro fluorescence measure-
ments. Promoter regions were amplified from purified Y. enterocolitica DNA by
PCR using the primers listed in Table 2. PCR products were digested with EcoRI and
SacI (MBI Fermentas, Vilnius, Lithuania), ligated (T4 DNA ligase; Gibco,
Hudson, N.H.) into the SacI/EcoRI site of pPROBE-NT (42) and transformed
into DH5αMCr. Plasmids containing the correct transcriptional gfp fusions as
verified by PCR were isolated (GenElute Plasmid Mini Prep Kit; Sigma-Aldrich,
Taufkirchen, Germany) and transformed into Y. enterocolitica. Bacterial cells bearing the recombinant gfp promoter probe vectors were grown in parallel at
30°C and 10°C in LB medium supplemented with kanamycin. From both cul-
tures, 5-ml samples were harvested at an OD_{600} of approximately 1.8, centrifuged
(6,000 rpm for 2 min at RT), shock frozen, and stored at −70°C. Pellets were
washed once with 500 μl phosphate-buffered saline, centrifuged again, and re-
suspended in 500 μl phosphate-buffered saline. Quantitative fluorescence assays
were performed using a Wallac VICTOR2 1420 multilabel counter (Perkin
Elmer Life Sciences, Turku, Finland) with sterile, untreated, black 96-well mi-
croplates (Nunc, Wiesbaden, Germany). Suspensions (each, 200 μl) were mea-
sured at wavelengths of 485 nm for excitation and 520 nm for emission. Intensity
readings are presented as relative fluorescence units.

**RESULTS**

Identification of 42 cold-responsive transcriptional units. Out of 5,700 transposon mutants, 4,454 (78%) mini-Tn5 lux
CDABE insertions into Y. enterocolitica strain W22703 showed a response ratio of <2.0 or an average light emission at back-
ground level of 200 RLUs and were classified as not differenti-
ally expressed at both temperatures. Finally, 1,238 clones
were identified to be steadily upregulated at 10°C with a
response ratio of at least 2.0. Although a twofold difference in
relative transcript levels is a threshold commonly used for
analysis and interpretation of microarray data (32), we used a
threshold of 5.0 to further analyze only mutants with strongly
elevated activity of the respective promoter. From the 222
mutants matching this criteria, about 40% showed light emis-
sion profiles congruent to those of mutants already identified,
indicating a redundant transposon insertion, or exhibited un-
steady, unrepeatable profiles with strong data fluctuation.
Those perturbations were especially derived from promoters with
transcriptional activity slightly above background. Another
11% were withdrawn because they were false positive,
due to neighboring mutants with strongly up-regulated light
production in the microtiter plate. In total, we selected 109
clones for further characterization, representing approximately
1.9% of the 5,700 transposon mutants, plus 5 clones as exam-
ple for strongly inhibited luciferase activity at low tempera-
ture. The DNA of those mutants for which we observed re-
sponse ratios of >5.0 or <0.6 and a steady expression pattern
was digested and used for inverse PCR as described. The

![TABLE 3—Continued](http://jb.asm.org/Downloadedfrom)
sequences of chromosomal fragments flanking the transposon insertion were compared to the Y. enterocolitica DNA assembly, and the results of the BLAST analysis allowed the precise localization of the transposon insertion and the orientation of the inserted reporter genes with respect to the Y. enterocolitica genome sequence, except for strain-specific fragments. This analysis revealed 42 different genes or open reading frames (ORFs) with enhanced (37 genes) or reduced (5 genes) promoter activity at 10°C compared to 30°C, 13 genes of which are probably localized within putative operon structures. This finding suggests that all proteins encoded by these putative operons are present in the cell in higher or lower copy numbers in response to low temperature. It should be noticed that the 37 genes represent all mutants characterized by a response ratio of the luciferase reporter of $H_{11022}^{5.0}$, whereas the five strains with down-regulated promoter activity are examples only of a larger group of mutants not further characterized. For two transposon insertions, no promoter responsible for luciferase induction could be identified, due to preliminary annotation of the respective genome region. An accumulation of independent transposon insertions was found within three ORFs or their surroundings, namely 13 insertions in both tcaB and tcaA and 10 insertions in a putative alkaline serine protease (YE2922). All transposon insertions are listed in Table 3.

No genes indispensable for growth at low temperature were identified. Given that the library is random and that the genome of Y. enterocolitica is composed of approximately 2,500 transcriptional units similar to E. coli (68), our library should allow testing of approximately 70% of all genes or operons for differential expression (38). For this calculation, we assumed that the mini-Tn5 luxCDABE insertion in 50% of the mutants was not under control of a promoter located upstream. Despite this high knockout fraction, none of the insertions affected a gene or gene complex that is dispensable for cell growth at 30°C but specifically required for growth at low temperatures. Of the tested mutants, 0.5% were growth deficient at 10°C, as well as at 30°C.

gfp reporter fusions validate gene identification. To prove the differential transcription of the identified genes, we cloned the first assigned or putative promoter regions located upstream of nine mini-Tn5 luxCDABE insertions and the promoter region of cspB as a control into vector pPROBE-NT and measured GFP expression at 30°C and 10°C. Since the autocatalytic protein modification required for fluorescent light emission by GFP proceeds slowly in bacterial cells (2), fluorescence values were taken at an OD$_{600}$ value of 1.8. The background activity derived from a nonrecombinant vector in Y. enterocolitica alone was 3.9-fold ($H_{11006}^{0.3}$) higher at 30°C than at 10°C, and this factor was used to normalize the derived gfp reporter activities at 10°C (Fig. 2). This factor describes the reduced synthesis rate of GFP, due to the temperature decrease, and correlates with the Arrhenius equation predicting a factor of 1.5 to 4.0 (57). In all cases but one, the investigated promoters were shown to be induced 1.1 to 102 fold with an absolute range of fluorescence units from 2,536 RFU (urease operon) to 3,119,696 RFU (flagellar operon) at 10°C. An exception was observed when a gfp fusion to a putative promoter region directly upstream of YE0480 was tested. However, fur-
ther annotation studies revealed that YE0480 is probably transcriptionally coupled to gene YE0479, a finding that might explain the negative result. In another cold-responsive mutant that we examined by GFP fusions, the luxCDABE genes had inserted between the ORFs YE0950 and YE0951, and a putative promoter upstream of YE0951 was found to be opposite to the direction of luxCDABE transcription. However, when the predicted promoter was amplified from this intergenic region and cloned into pPROBE-NT in correct orientation, a 2.4-fold induction of GFP at low temperature was observed (Fig. 2). This result and promoter prediction studies suggest that the selected promoter directs the polycistronic transcription of five genes (YE0951-yeuB-ureC-ureE-ureF) involved in urea metabolism. For all but one promoter probe constructs, the response ratio of gfp was significantly lower than the values deduced from the bioluminescence signal. These discrepancies are probably due to differences in both assays in copy numbers of reporter genes, half-lives, number of genes involved in light emission, reporter sensitivity, and experimental design.

**Low temperature-responsive genes of Y. enterocolitica.** The kinetic data derived from the light emission of transposon recipients grown at 30°C and at 10°C revealed a pattern of varying gene expression profiles due to mini-Tn5 luxCDABE insertions under the control of temperature-responsive promoters (Fig. 3). The interpretation of these profiles allowed (i) determination of luciferase induction with respect to the OD_405 value, (ii) quantification of the transcriptional response to low temperature by the normalized response ratios of luciferase fusions, and (iii) qualification of the induction as transient or permanent for a certain time interval. The response ratios of the mentioned genes were ranged from 6 to 1,147 (Table 3), with the maximal induction of a hypothetical protein (YE4063) that was not transcribed at 30°C and showed pronounced similarity (E value, 10^{-24}) to conserved domains of
signaling during proteins. A summary of the most important genes induced during long-term response of \textit{Y. enterocolitica} is provided in Fig. 4. In contrast to cold shock proteins (CSPs), several transcriptional profiles showed no clear peak but steadily increased after temperature downshift and remained at a higher expression level, e.g., the mutants YE2057::\textit{lux CDABE} and \textit{uhpC}::\textit{lux CDABE} (Fig. 3B and I). On the other hand, the change in promoter induction was only transient in transposon mutants like YE2537::\textit{lux CDABE} (Fig. 3C), indicating a maximal expression of the basal body M-ring protein at an OD\textit{t} of 0.8.

The genes of 37 transcriptional units significantly up-regulated during growth at 10°C were grouped into three classes according to the growth phase at which transcriptional induction was detected. At acclimatization phase (OD\textit{t} < 0.3), two transposon insertions were located within the genes encoding CspA and CspB, showing a strong normalized response ratio of 84 and 268 after cold shock. CspA was transiently expressed with a peak 4 h after temperature downshift (Fig. 3J), while light emission driven by the \textit{cspB} promoter decreased only slightly during prolonged growth (Fig. 3E). The light emission profile of the \textit{cspA::lux CDABE} mutant correlated with Northern blot analysis, showing inhibited \textit{cspA} transcription 120 min after cold shock (46) and with the relative amount of the major CSPs as determined by two-dimensional gel electrophoresis (Fig. 3J) (45), thus demonstrating the validity of chromosomal \textit{lux CDABE} promoter fusions as a tool to measure transcriptional activities. The temporal distance between the maximal amount of CspA and the corresponding luciferase activity was calculated as 180 to 195 min. The sustained luciferase activity of the \textit{cspB::lux CDABE} knockout mutant might be explained by an autoregulatory effect known to occur for \textit{E. coli cspA} (4). Parallel to both CSPs, mini-Tn5 \textit{lux CDABE} insertions within the promoter region of \textit{ymaA}, which plays a role in thermoregulation of virulence functions (15), and within the ORF of \textit{gltP}, encoding a glutamate-aspartate symport protein, were shown to be upregulated within the acclimatization phase.

Early and mid-exponential growth phase \textit{(0.3 < OD\textit{t} < 0.8)}. Seventy percent of the transcriptional units identified in our approach were induced at 10°C before they reached the late growth phase, most of them involved in sensing-regulation, motility, and virulence. Besides several as-yet-uncharacterized genes, we found \textit{arcA} (YE0595), which is necessary to survive prolonged starvation (59), \textit{ubpABC}, controlling the expression of the hexose phosphate transporter \textit{UhpT} (70), and two obviously strain-specific loci without homology to known genes. The most prominent response to temperature decrease was the enhanced transcription of motility and chemotaxis genes such as the flagellin operon or \textit{cheA}.

Differentially expressed genes are YE2057, which encodes a homologue of the putative virulence factor SrfA of \textit{Salmonella}, \textit{mgfC} (YE2586) involved in magnesium uptake (44, 65), and YE2463 encoding an outer membrane porin. Surprisingly, we identified two mini-Tn5 \textit{lux CDABE} insertions in the genes \textit{tcaA} and \textit{tcaB} encoding two subunits of Tca, a homologue of the insecticidal toxin complex proteins from \textit{Photorhabdus luminescens}, and we measured response ratios of 12 and 81 when cultivating these mutants at 10°C (11). We also observed the induction of two putative operons involved in urease activity. One was YE0951-yebB-\textit{ureC}-\textit{ureE-ureF}, encoding urea amidohydrolase, an accessory protein, and three urease subunits. The other operon was YE0959-YE960, which encodes a urease accessory protein and a urea transporter.

Late exponential and early stationary growth phase \textit{(OD > 0.8)}. During late exponential growth, we observed the stimulation of the following genes: YE0480, encoding a predicted hemolysin transporter with homology to the accessory protein FlhC from \textit{Bordetella pertussis} (identity, 28%; \textit{E} value, 2\textsuperscript{-45}), the monocistronically organized gene YE2922, encoding a homologue of subtilisin (identity 33%; \textit{E} value, 6\textsuperscript{-28}), which is an alkaline serine protease associated with the onset of sporulation in \textit{B. subtilis} (67); two genes encoding a histidine ammonia-lyase (\textit{luxH}) and a putative amylase (YE4013); and one gene (YE3697), encoding a permease of the drug/metabolite transporter (DMT) superfamily.

Down-regulated promoters. Due to its high dynamic range, the luciferase reporter also allows the assessment of decreased transcriptional activity in long-term response to temperature changes (75). We mapped five mini-Tn5 \textit{lux CDABE} insertion mutants responding to growth at 10°C with strong reductions in luciferase activity. One of these insertions was found to be located within gene YE4164, encoding a protein identical to heat shock proteins of the \textit{IbpA} family, probably cotranscribed with YE4163, which encodes an \textit{IbpB}-like heat shock protein. At 10°C, very low luciferase activity corresponding to the background level was observed for the putative promoters in front of gene YE4121, which encodes a sugar-binding periplasmic protein, and upstream of YE3774, which encodes a putative phosphotrehalase as part of a phosphotransferase system. This is in line with the observed elevated transcription levels of \textit{cheA} at low temperature, resulting in a tumbling behavior of bacterial cells. The activity of the phosphotransferase system, however, that transports carbohydrate attractants into the cell is known to extend swimming runs (40). Interestingly, the sequence obtained from another transposon insertion was identical to an as-yet-unannotated region of the \textit{Yersinia virulence} plasmid pYE8081 immediately downstream of \textit{yopT/ysycT}, and the maximal light emission of 20,489 RLU/OD obtained for the respective mutant at 30°C was reduced by 1 order of magnitude to 3,865 RLU/OD (Table 3).

Inhibitory effect of \textit{UhpC} and \textit{MCPI} on the expression of YE0480. The above-described determination of induction time points of gene expression should allow potential interdependencies between the cold-responsive genes found to be uncovered. To examine the effect of knockouts of three regulatory genes from Table 3 on selected transposon mutants, insertional duplication mutagenesis of the three regulatory genes \textit{uhpC, ybcM}, and YE2575 was performed with a set of 24 temperature-responsive \textit{lux CDABE} mutants, resulting in a total of 72 double mutants. None of the insertion mutants showed deficient growth at 10°C or 30°C, indicating that the double knockout did not affect cell viability (data not shown). The promoter activities of the double mutants were measured at 10°C and 30°C, and the data were compared to the bioluminescence values of the corresponding mutants without additional insertional knockout mutations. In most cases, this analysis did not reveal significant changes in promoter activities of the double knockout mutants in comparison to the single \textit{lux CDABE} insertion mutants. However, two exceptions were observed: the activity of the \textit{lux} reporter inserted in YE0480, encoding an
FhaC-like transporter, was enhanced by the factor 4.8 and 5.6 at 15°C as a result of the insertional knockouts of YE2575 and uhpC, respectively, and by a factor of approximately 10 at 30°C (Fig. 5). The cloned uhpC and YE2575 genes complemented the mutations; in the case of uhpC, the light signal of the complemented strains exceeded that of W22703-YE0480::lux, probably due to the lack of the native uhpC promoter. These results indicate that the two-component system UhpABC, as well as methyl-accepting chemotaxis protein I (MCPI) involved in chemotaxis, negatively regulates the expression of the putative hemolysin transporter encoded by YE0480.

FIG. 5. Inhibitory effect of the insertional knockout of uhpC and YE2575 encoding MCPI on the transcription of YE0480::luxCDABE at 30°C and 15°C. Luciferase activities are shown in corrected RLU. The data represent the means and standard errors of means for three independent measurements.

DISCUSSION

Cold acclimatization versus long-term adaptation. In many cases, gene induction has been studied immediately after sudden changes, e.g., cold shock, acid shock, or heat shock, while much less effort has been dedicated to analyzing gene expression during prolonged growth under a specific environmental stress. It is a significant result of this work that there is no overlap of the well-described cold acclimatization response directly following cold shock and the genes induced during exponential growth at low temperature. This is supported by our negative results of an in silico search for consensus regulatory regions within promoter regions or translational start regions of cold shock genes, e.g., the putative downstream box or the cold box (22, 43). Similar observations have been reported for the UV-B shock response and subsequent growth under elevated UV-B of the cyanobacterium Nostoc commune (19), and for L. monocytogenes grown to mid-exponential phase (39). The pronounced separation between shock response and long-term adaptation may be a general feature of bacterial gene expression induced by environmental stress.

Y. enterocolitica virulence factors at environmental temperature. At least two virulence factors of Y. enterocolitica have been known to be maximally expressed at ambient temperatures, the heat-stable enterotoxin Yst (10) and the outer-membrane protein Inv (53). One of the most important aspects of differential gene expression in terms of the Y. enterocolitica temperature response that emerged from our study is the enhanced transcription of another four genes associated with virulence functions. At low temperature, we observed the up-regulation of tcaA and tcaB encoding homologues of the insecticidal toxin subunits from P. luminescens that is found in the gut of entomopathogenic nematodes (72). Extracts of Y. enterocolitica grown at 10°C but not 30°C were shown to be lethal for insect larvae, and tcaA was demonstrated to be essential for this insecticidal activity. This finding gave rise to speculations about an as-yet-undisclosed niche of Y. enterocolitica in insects (11). The transcription of another putative virulence factor, a homologue of the SsrAB-regulated factor SrfA (76), was also shown to be low-temperature responsive. This is in line with the observation that srfA transcription is repressed fivefold during intracellular growth in macrophages at 37°C (21). Interestingly, microarray data of Salmonella ty-
phimurium motility only recently revealed the surface-dependent regulation of several virulence genes, among them srfABC, thus demonstrating the association between swarming behavior and virulence, as observed with many bacteria (28, 71). YE0480 is possibly involved in hemolysin secretion, and hemolysins are predicted determinants of Photorhabdus pathogenicity against insects (12). It is remarkable that neither tcaA, tcaB, srfA, nor YE0480 showed significant transcriptional activity at 37°C (data not shown). The correlation of bacterial pathogenicity with exponential phase is reminiscent of a molecular model that describes factors involved in the association of Photorhabdus with nematodes and insects (24). Taken together, these data suggest a role of these virulence factors outside mammalian hosts, possibly in invertebrates.

Environmental sensing and motility. The specific expression profile at low temperature discussed here strongly supports the view of temperature as an important stimulus for the long-term adaptation of Y. enterocolitica to environmental changes, mediated by sensory proteins and regulatory networks. In our approach, we observed the induction of several regulatory systems of known (ArcA and UhpABC) and predicted (YE1436, ybeM, YE4063, and YE1324) function, many of them involved in signal transduction (Table 3). The ArcAB system controls the expression of at least 40 operons involved in catabolic gene expression (3, 59), and arcA is suggested to control the rate of utilization of endogenous reserves (49). None of our results points to the presence of a cold regulon during prolonged growth at 10°C. However, YE0480, a gene encoding a homologue of the accessory protein FhaC from B. pertussis, is strongly expressed at 10°C but not at 37°C, and its transcription is negatively influenced by MCPI and UhpC, suggesting that YE0480 expression is connected with nutrient acquisition and motility regulons. Interestingly, a similar regulatory circuit was reported recently for expression and activity of a Xenorhabdus nematophila hemolysin that is required for full virulence against insects (16). These interdependencies confirm the linkage between motility and virulence properties at the level of regulation (18). The induction of flagellae and chemotaxis genes at low temperature is a well-characterized phenomenon that is in line with the finding that loss of motility at 37°C does not affect pathogenesis of yersiniae in humans (33), indicating a role of motility in the colonization of new habitats. The master flagellum regulatory operon, flhDC, is assumed to be required for full Xenorhabdus virulence against insects. Moreover, the flagellar sigma factor of Y. enterocolitica, α28, controls the phospholipase gene yplA, whose product is exported by the flagellum secretion apparatus (58).

Degradative metabolism: further evidence for an insect stage during the Y. enterocolitica life cycle. A predominant feature of the derived expression profile at environmental temperatures is the prevalence of genes involved in biodegradative metabolism during late growth and stationary phase. We found the significant upregulation of two urea operons and of a histidine ammonia-lyase (YE3021) with homology to the HutH histidase, all of them involved in nitrogen utilization. In addition, low temperature results in elevated expression of the α-glucan branching enzyme GlgB (YE4013) that, together with the α-amyase GlgX and GlgC, regulates the glycan metabolism (56). Interestingly, the degradative hut operon, as well as an α-amyase, has been shown to be upregulated at low temperature in severalantarcticpsychrotrophicbacteria (23, 36). A role of the Y. enterocolitica alkaline serine protease, a hot spot of mini-Tn5 luxCDABE insertions and a member of the extracellular subtilisin family (67), has not yet been described. Proteases, however, represent exoenzymes that might help to degrade tissues and macromolecules of a eukaryotic host. None of the genes mentioned as being involved in degradative metabolism is expressed at 37°C (data not shown), again suggesting a role of these enzymes outside a mammalian host. It is therefore tempting to speculate that these genes play a role in bioconversion of an insect cadaver, providing a nutrient base for bacterial growth. Taken together, the long-term portrait of cold-responsive genes as derived from the luciferase assay is a promising starting point for further analysis of the association of Y. enterocolitica with invertebrates.

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