Regulation of the Dha Operon of *Lactococcus lactis*

*A DEVIATION FROM THE RULE FOLLOWED BY THE TetR FAMILY OF TRANSCRIPTION REGULATORS*[^1][^2]

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Dihydroxyacetone (Dha) kinases are a novel family of kinases with signaling and metabolic functions. Here we report the x-ray structures of the transcriptional activator DhaS and the coactivator DhaQ and characterize their function. DhaQ is a paralog of the Dha binding Dha kinase subunit; DhaS belongs to the family of TetR repressors although, unlike all known members of this family, it is a transcriptional activator. DhaQ and DhaS form a stable complex that in the presence of Dha activates transcription of the *Lactococcus lactis dha* operon. Dha covalently binds to DhaQ through a hemiaminal linkage which is propagated to the surface via a cantilever-like structure. DhaS binds to its promoter, which is protected by Dha binding site, and thereby induces a conformational change. Dha kinase subunits, DhaK and DhaL are corepressor and coactivator of DhaS. When Dha is present and phosphorylated, DhaL-DhaK subunit binds Dha covalently by a hemiaminal linkage to Dha, which in contrast to the nucleotide of the ATP-dependent kinases is not exchanged but is rephosphorylated. This allows the phosphorylation of Dha via DhaM. DhaM is homologous to the IIAmannosyltransferase subunits of the PTS system. The enzymes of metabolic pathways have by and large been conserved in all kingdoms of life where they occur. In contrast, the mechanisms of pathway control are diverse.

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[^2]: The on-line version of this article (available at http://www.jbc.org) contains supplementary materials including Tables S1–S3 and Fig. S1.

[^3]: The atomic coordinates and structure factors (code 2IU4, 2IU5, and 2IU6) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

[^4]: The abbreviations used are: Dha, dihydroxyacetone; PEP, phosphoenolpyruvate; PTS, PEP-dependent carbohydrate phosphotransferase system; DhaS, transcription activator of the dha operon; DhaQ, coactivator of DhaS; DhaKin and DhaH, subunits with C-terminal histidine tag; DhaK, Dha binding subunit of the *E. coli* Dha kinase; DhaL, nucleotide binding subunit; DhaM, phosphotransferase subunit (Dha kinase-specific phosphotransfer protein of the PTS); NTA, nitritolactate acid; EMSA, electrophoretic mobility shift assay; RMS, root mean squared.

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The enzymes of metabolic pathways have by and large been conserved in all kingdoms of life where they occur. In contrast, the mechanisms of pathway control are diverse. This is most obvious at the level of gene expression. The different size, structure, and sequence organization of eukaryotic and prokaryotic genomes necessitate the different control mechanisms. But even between bacteria with similar genome organizations the differences can be striking. The transcription control of dihydroxyacetone kinases is one example for such diversity, as it will be shown below.

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Dihydroxyacetone (Dha)[^4] kinases occur in eubacteria, animals, and plants. They can be divided into two families according to the source of high energy phosphate they utilize, ATP and phosphoenolpyruvate (PEP) (for a review see Ref. 1). The ATP-dependent kinases from animals, plants, and eubacteria consist of a Dha binding and an ATP binding domain. The PEP-dependent forms consist of three protein subunits DhaK, DhaL, and DhaM (2). DhaK and DhaL are homologous to the Dha ATP binding domains and DhaM is homologous to the IIAmannosyltransferase subunits of the PTS system (3, 4). DhaK is a stable homodimer of 35-kDa subunit molecular mass that binds Dha covalently by a hemiaminal linkage to the imidazole nitrogen of a histidine (His-230 in *Escherichia coli*) and the carbonyl carbon of Dha (5, 6). DhaL contains a molecule of ADP, which in contrast to the nucleotide of the ATP-dependent kinases is not exchanged but is rephosphorylated in situ by DhaM (7). DhaM shuttles phosphate from the phosphorylcarrying E. coli receptor to DhaL.

A BLAST analysis with DhaK and DhaL as query revealed genes for DhaK and DhaL homologs, which were associated in operons with the genes for putative transcription factors (1). These genes occur adjacent to the Dha kinase operons suggesting that they control Dha kinase expression. How this works has so far been elucidated only for *E. coli* (8). Here, the *dha* operon is controlled by DhaR, a transcription activator from the family of AAA⁺ enhancer-binding proteins (EBP, Ref. 9). The Dha kinase subunits, DhaK and DhaL are corepressor and coactivator of DhaR. When Dha is present and phosphorylated, DhaL-ADP is formed, which by binding to the DhaR receiver domain stimulates DhaR activity. In the absence of Dha, the ligand-free DhaK subunit (apoDhaK) displaces DhaL from the receiver domain but unlike DhaL does not activate DhaR (8). The transcription control of the *dha* operon is thus coupled to the enzymatic turnover of the inducer rather than to binding of the inducer alone. This is one example to show how Dha kinase subunits sense Dha and induce their own expression. A second example is provided by the *dha* operon of *Lactococcus lactis*...
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IL1403 that has not been characterized before. This operon (Fig. 1A) for a putatively PEP-dependent Dha kinase and a glycerol-type facilitator is flanked by two genes dhaS and dhaQ. Here we present the x-ray structures of the two subunits DhaS and DhaQ and characterize their function. DhaS belongs to the TetR family of transcription regulators (6). But unlike all known members of this family DhaS of L. lactis functions as an activator and not as a repressor of transcription. DhaQ is a paralog of the DhaK subunit, which in complex with Dha acts as the coactivator of DhaS.

EXPERIMENTAL PROCEDURES

Crystallization, Structure Determination, and Refinement of DhaS—For details of protein expression and purification see the supplemental text. Crystals of DhaSH6, a variant of DhaS with a C-terminal histidine tag, were obtained by sitting drop vapor diffusion at 20 °C. The concentrated protein (20 mg/ml) was mixed with buffer containing 50 mM sodium cacodylate pH 6.0, 1.7 M ammonium sulfate, 0.015 M magnesium acetate in a 1:1 ratio. For cryoprotection, 25% glycerol was added in a serial manner. Crystals were frozen in liquid nitrogen. Mercury derivatives with different occupancies were obtained by soaking in 1 mM HgCl2 for three months (derivative 1) and in 0.1 mM HgCl2 for 12 h (derivative 2). To increase the phasing power the selenomethionyl L171M mutant of DhaSH6 was used as an additional derivative. The datasets for the mercury derivatives 1 and 2 and the L171M selenomethionyl derivative were collected at 110 K using a RAXIS-IV imaging plate detector mounted on a Rigaku RU-300 rotating anode x-ray generator (wavelength 1.5418 Å). High resolution diffraction data for the native crystal were collected at the BM14 beamline (wavelength 0.976 Å) of the European Synchrotron Radiation Facility (Grenoble, France) employing a MAR Mosaic225 detector (Mar Research, Hamburg, Germany). All datasets were integrated and scaled by XDS (14, 15). The structure was determined by the MIRAS method. Heavy atom (mercury and selenium) sites were located by SHELXD (10) and difference Fourier methods. Phasing and solvent flipping were effected using SHARP (11) and SOLOMON (12) resulting in a figure of merit of 0.66 to 2.31 Å resolution for the unmodified map. Automatic model building was achieved using the program ARP/WARP version 6.1.1 (13). The model was further adjusted and completed using the program O (14) version 9.0.3. Refinement was effected by the program REFMAC (15). The quality of the model was checked by PROCHECK (16). The results of the data collection and processing statistics are given in Table 1. The asymmetric unit of the crystal contains two DhaS monomers (one physiological dimer). The final refined coordinates consist of 178 and 179 residues in the two subunits. More than 95.7% of the residues of the final model fall within the core region and 4.3% of the residues in the allowed region of the Ramachandran plot. Atomic coordinates and structure factor amplitudes have been deposited with the RCSB, with accession codes 2IU4, and 2IU5. Comparison of the three-dimensional structures was done using the DALI server (17).

Crystallization, Structure Determination, and Refinement of apoDhaQ and the Dha-DhaQ Complex—Crystals of apoDhaQ were obtained after several weeks by mixing 2 μl of protein (15 mg/ml) with 2 μl of 0.15 M L-alanine, 0.1 M sodium acetate trihydrate, pH 4.5, 2 M ammonium sulfate. Crystals of the Dha-DhaQ complex were obtained in 0.1 M sodium acetate trihydrate, pH 4.5, 2 M ammonium sulfate. Crystals were cryoprotected by adding glycerol to 25%. The complete datasets of apoDhaQ were collected in-house (wavelength 1.5418 Å) and of the Dha-DhaQ complex at the Swiss Light Source (wavelength 1.2154 Å), PSI, Villigen.

The structure of apoDhaQ was solved by molecular replacement using the DhaK structure from E. coli (PDB code 1OI2) and employing the program PHASER (18). The density was further improved by CNS and an initial automated protein model was constructed into the electron density using the program ARP/WARP. The results of the data collection and processing statistics are given in Table 2. The asymmetric unit of the crystal contains two DhaQ monomers (one physiological dimer). 90.3% (90.2%) of the residues of the final apoDhaQ (Dha-DhaQ complex) model fall within the core region and 8.8% (8.9%) in the allowed region of the Ramachandran plot. Atomic coordinates and structure factor amplitudes have been deposited with the RCSB, with accession code 2IU6.

Determination of β-Galactosidase (LacZ) Activity—L. lactis IL1403 transformed with pDKQ5, pDKS, and pDK (Fig. 1B) were used for the reporter gene assay. β-Galactosidase activity was determined by the method of Miller (19, 20). Cultures were grown in M17 medium (Difco) (supplemented with 0.5% glucose, 5 μg/ml chloramphenicol, and 5 mM Dha where indi-

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5 The amino acid sequence of the proteins can be accessed through Swiss Protein Database Swiss-Prot Q9CIV0 (DhaQ), Swiss-Prot Q9CIV9 (DhaS), Swiss-Prot Q9CIV8 (DhaK), Swiss-Prot Q9CIV7 (Dhal), and Swiss-Prot Q9CIV6 (DhaM).
TABLE 1

Data collection, phasing, and refinement statistics for DhaS (MIRAS)

|                           | Native | HgCl₂-D 1 | HgCl₂-D 2 | SeMet |
|---------------------------|--------|-----------|-----------|-------|
| **Data collection**       |        |           |           |       |
| Space group               | P2₁2₁₂₁|           |           |       |
| Cell dimensions           |        |           |           |       |
| α, β, γ (Å)               | 44.46, 46.28, 171.94|           |           |       |
| Resolution (Å)            | 4·1.6 (1.65–1.6) | 4·2.26 (2.39–2.26) | 4·2.39 (2.55–2.39) | 4·2.26 (2.39–2.26) |
| Rmerge b                  | 5.3 (18.3) | 4.4 (7.8) | 5.2 (11.9) | 3.8 (7.3) |
| I/σI                      | 16.0 (7.2) | 32.3/12 | 18.6 (9.6) | 29 (10.9) |
| Completeness (%)          | 99.2 (100) | 99.3 (96.3) | 91.9 (63.1) | 89.7 (32.5) |
| Redundancy                | 4·4 (4.01) | 4·9 (2.45) | 3·17 (3.11) | 4·45 (2.12) |
| **Refinement**            |        |           |           |       |
| Resolution (Å)            | 40·1.60 |           |           |       |
| No. reflections           | 45492 |           |           |       |
| Rmerge/Rfree              | 19.5/23.0 |           |           |       |
| **No atoms**              |        |           |           |       |
| Protein                   | 3034 |           |           |       |
| Water                     | 297 |           |           |       |
| **B-factors**             |        |           |           |       |
| Protein                   | 22.70 |           |           |       |
| Water                     | 30.20 |           |           |       |
| **R.m.s. deviations**     |        |           |           |       |
| Bond lengths (Å)          | 0.008 |           |           |       |
| Bond angles (°)           | 1.682 |           |           |       |

*One crystal was used for each data set.

b Highest resolution shell is shown in parentheses.

dicated) to an A_{250} of 0.4–0.6 and permeabilized with one drop of toluene and 30 s vortexing. The toluene was evaporated, the permeabilized cells incubated for 5 min at 28 °C, o-nitrophenyl-β-D-galactopyranoside (30 μM of 4 mg/ml in buffer Z to 110 μM of cells) was added to start the reaction, absorbance at 420 nm was monitored continuously in a Spectramax 250 Plate reader and converted into Miller units (MU).

For primer extension total RNA was isolated from L. lactis IL1403 with the Nucleo Spin RNA II Kit (BD Biosciences). The primer P13 annealing to the codons 13–19 of dhaK was 32P end-labeled, and the extension reaction was performed according to the primer extension system protocol of Promega. The primer extension product was analyzed on a 6% denaturing acrylamide gel.

RESULTS AND DISCUSSION

Structure Determination and Overall Architecture of DhaS—DhaS with a C-terminal hexahistidine tag was expressed in E. coli BL21(DE3) and purified by metal affinity and gel filtration chromatography. The final yield was 35 mg of DhaS per liter of culture (for details, see supplemental information). Details of structure determination and refinement statistics at 1.6 Å resolution are given in Table 1. The asymmetric unit contains two monomers of DhaS forming a wedge-shaped physiological dimer. Both DhaS monomers are well ordered (residues 1–179) except for the first three amino acids in the second and the last amino acid in the first monomer.

The DhaS monomer folds into nine α-helices (α1–α9) (Fig. 2A). The DhaS monomer can be divided into an N-terminal DNA binding domain (α1–α3) and a core domain involved in dimerization (α4–α9). The N-terminal helices α2 (residues 25–35) and α3 (residues 40–45), which are almost perpendicular to each other, form the helix-turn-helix DNA binding motif. Helices 5 and 6 (residues 75–99) and helix 7 (residues 132 to 156) and helix 8 (residues 132 to 156) and helix 9 (residues 161–178) provide a dimerization interface of 1090 Å² per monomer, which covers 11% of acrylamide-bisacrylamide 19:1, 150 V, 0.7 h) and visualized by exposure on a phosphor screen.
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The subunit surface. It contains 65% hydrophobic, 22% polar and 13% charged amino acids.

As indicated by its fold, DhaS belongs to the TetR family of transcriptional regulators (21). A sequence alignment of DhaS with the Staphylococcus aureus multidrug regulator QacR (22, 23) and the E. coli tetracycline repressor TetR (24, 25) shows invariant residues predominantly in the DNA binding domain (α1–α3) whereas no sequence conservation was found in the core domain. In particular, the residues in positions critical for inducer recognition by the TetR family members (21) are not conserved in DhaS. Overlay of the DhaS and QacR monomers yields an RMS deviation of 4.0 Å for 161 out of 186 Ca, overlay with TetR a rms deviation of 4.2 Å for 134 of 198 Ca (17). An overlay of the physiological dimers is shown in Fig. 2B. DhaS has a more open core fold and as a consequence the helix-turn-helix motifs are further apart in DhaS than in QacR. The distances between the Ca and Ca’ of Tyr-44 is 63 Å in the DhaS dimer, whereas it is 45 Å in QacR and 37 Å in TetR (not shown). The increased distance between the DNA binding domains of DhaS is caused by the following differences (relative to QacR) (i) different orientations of the core helices, (ii) different orientation of the DNA binding domain relative to the core helix 4, (iii) different angles between the helix axis of α1 (DNA binding domain) and α4 (core), namely −120° in DhaS but only −90° in QacR.

Structure Determination of ApoDhaQ and the Dha-DhaQ Complex—DhaQ with a C-terminal hexahistidine tag was expressed in E. coli BL21(DE3) and purified by metal affinity and gel filtration chromatography. The final yield was 60 mg of DhaQ per liter of culture (for details, see supplemental information). The structures of the apo-form of DhaQ and the Dha-DhaQ complex were solved by molecular replacement, the apo-form employing the DhaK subunit of E. coli as a search model (6), and the complex using the refined apo-model. Refinement and data collection statistics are given in Table 2. The asymmetric units of both crystal forms contain one physiological dimer. The Ca backbone of the Dha-DhaQ complex is well ordered over its entire length (residues 8–332 and C1, C2, and C3 are hydrogen-bonded to the side chain of Asp-Gly-51, respectively (Fig. 3A) whereas no sequence conservation was found in the active site could be assigned to a molecule of glycerol (Fig. 3A).

Table 2 Data collection and refinement statistics for apoDhaQ and Dha-DhaQ (molecular replacement)

|                         | ApoDhaQ       | Dha-DhaQ     |
|-------------------------|---------------|--------------|
| Space group             | P3,21         | P3,21        |
| Cell dimensions (Å)     | 101.09, 101.09, 148.60 | 99.9, 99.9, 188.57 |
| α, β, γ (°)             | 90, 90, 120   | 90, 90, 120  |
| Resolution (Å)          | 40.2.0 (2.04-2.00) | 40.196 (2.04-1.96) |
| Rmerge (°)              | 8.5 (45)      | 10.2 (28.9)  |
| l(Å)                    | 22.6 (6.88)   | 11.17 (5.66) |
| Completeness (%)        | 100 (100)     | 99.9 (99.9)  |
| Redundancy (°)          | 12.38 (10.95) | 5.39 (4.54)  |
| **Refinement**          |               |              |
| Resolution (Å)          | 30.2.00       | 30.1.96      |
| No. reflections         | 58399         | 76689        |
| Rwork/Rfree             | 17.30/19.8    | 18.1/20.9    |
| **No. atoms**           |               |              |
| Protein                 | 4981          | 5098         |
| Ligand/ion              | Glycerol (12) | Dha (12)/Sulfate (20) |
| Water                   | 278           | 338          |
| **B-factors**           |               |              |
| Protein                 | 30.80         | 29.54        |
| Ligand/ion              | 31.56         | 29.49, 34.55 |
| Water                   | 34.55         | 34.38        |
| **R.m.s. deviations**   |               |              |
| Bond lengths (Å)        | 0.013         | 0.009        |
| Bond angles (°)         | 1.66          | 1.524        |

One crystal was used for each data set.

Highest resolution shell is shown in parentheses.

The asymmetric units of both crystal forms contain one physiological dimer. The Ca backbone of the Dha-DhaQ complex is well ordered over its entire length (residues 8–332 and 4–332). In the apo-form, however, residues 190–206 and 193–205 are disordered (Fig. 3B). The DhaQ monomer can be divided into two domains (Fig. 3A) each composed of a six-stranded sheet (yellow), which is covered by α-helices (blue for the first domain, red and green for the second domain). Helices 1 of the first and 9 of the second domain form the dimer interface. An area of 2400 Å² (27%) of the accessible monomer surface is buried upon dimer formation. Inserted in the second domain is a cantilever like structure consisting of two short β-strands (red, β10 and β11) from which a loop bearing the active site His-215 is suspended (Fig. 3A).

In the structure of apoDhaQ, extra electron density in the active site could be assigned to a molecule of glycerol (Fig. 3C), which is present in the crystallization buffer. The OH groups at C1, C2, and C3 are hydrogen-bonded to the side chain of Asp-107, Ne2 of His-54, and the main chain amide-hydrogen of Gly-51, respectively (Fig. 3C). In the Dha-DhaQ complex electron density can be assigned to Dha (Fig. 3D). The OH groups at
C1 and C3 are hydrogen-bonded to the side chain of Asp-107 and the main-chain amide-hydrogen of Gly-51, respectively. The geminal amino alcohol group at C2 is hydrogen bonded to Ne2 of His-54. The carbon backbones of the non-covalently bound glycerol and the covalently bound Dha backbone as well as the hydrogen bonding residues at the topological switch points of the first domain are perfectly superimposable (Fig. 3E). Dha binding initiates a series of movements and conformational rearrangements in and around the active site (Fig. 3, B and E): (i) His-215 is pulled by 1.37 Å toward Gly-51 and by 2.33 Å toward Asp-107. (ii) The imidazole ring of His-215 is rotated around the Cβ-Cγ bond, and as a consequence of this movement the hydrogen bond between N81 and the carboxyl Oe1 of Glu-217 is broken. The side chain of Glu-217 is rotated away and the free space is occupied by a sulfate ion, which is present in the crystallization buffer (Fig. 3E). (iii) Following the movement of His-215, the cantilever (red, β10 and β11) is pulled toward the N-terminal domain (blue helices in Fig. 3, A and B). (iv) The residues 190–206 and 193–205 that are disordered in apoDhaQ become well ordered upon Dha binding. An extra β-sheet appears as a result of this ordering (β8 and β9, Fig. 3, A and B). (v) The residues 134–140 of the N-terminal domain change their conformation from aperiodic to α-helical (Fig. 3B). All structural alterations appear to critically depend on the covalent binding of Dha, because the non-covalently bound glycerol has no effect. Soaking with Dha makes apoDhaK crystals crack, suggesting that conformational changes are induced by the ligand and that the different space groups assumed are a consequence of the different conformations and not vice versa. For comparison, none of these differences has been noticed with E. coli DhaK (33% sequence identity). In both forms (compare PDB ID 1OI2 and 1OIB, Refs. 6 and 26) the residues corresponding to 190–206

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**FIGURE 3. Structure of L. lactis DhaQ.** A, schematic representation of the DhaQ dimer in complex with Dha. One chain is shaded in gray, the other in color. The helices of the N-terminal domain and C-terminal domain are colored blue and green, respectively and numbered. The core β-sheets of both domains are colored yellow. The cantilever comprising β10 and β11 (italics) and the putative contact helix 7 are shown in red. The active site residues His-54 and Asp-107 (in the N-terminal domain) and His-215 (in the cantilever of the C-terminal domain) are shown as red sticks. B, stereo representation of the structural alignment of the Dha·DhaQ complex (red) and apoDhaQ (blue). The aperiodic to α-helical transition (residues 134–140), the disorder to order transition (residues 190–206, β-sheet), and the displacement of the cantilever (residues 207–224) are indicated with arrows. His-215 and glycerol in the apo-form and the His-215-Dha hemiaminal are shown as blue and red sticks. C and D, stereo pictures of experimental 2F̄ − F̄ electron density maps contoured at 1.0 σ of the apoDhaQ active site with non-covalently bound glycerol (C) and of the Dha·DhaQ complex with Dha in hemiaminal linkage to His-215 (D). E, active site with superimposed Dha·DhaQ (yellow) and apoDhaQ glycerol (CPK, gray). Hydrogen bonds are indicated as gray dotted lines. F and G, surface representation of multiple aligned DhaQ (F) and DhaK (G). Highly conserved regions are colored blue, intermediate cyan, and non-conserved green, yellow, and red. Notice, that the surface exposed face of helix 7 is strongly conserved in DhaQ but not in DhaK, while the entrance to the Dha (white arrow) binding site is better conserved in DhaK. The two models were built with CONSURF (28) using as input the six non-redundant DhaQ and DhaK sequences (supplemental Fig. S1), respectively, and the DhaQ structure as query. The orientation is 30° rotated around the x-axis relative to the model shown in A.
The structures of the low copy transcription factor (TetR) (Fig. 2), and DhaQ is a paralogous DhaK kinase subunit of L. lactis does not form a complex with DhaS pointing to the specificity of the DhaQ-DhaS interaction (Fig. 5A, inset).

The genetic analysis (above) suggested that Dha acts as the inducer of gene expression most likely through binding to one of the two subunits. To confirm this expectation, DhaQ was incubated with increasing concentrations of [14C]Dha and the complex then precipitated with acetone (5). A representative binding curve is shown in Fig. 5B. Binding reaches saturation at 5 μM Dha per 8 μM DhaS. Although less than one this stoichiometry is compatible with one Dha binding site per DhaQ monomer shown by the x-ray structure (Fig. 3). The Dha concentration for half-maximal saturation of DhaQ (Kd) was 14 ± 3 μM. For comparison, DhaK of E. coli which was used as a control in these experiments has a Kd of 0.8 μM. DhaS did not bind [14C]Dha (results not shown).

These observations, namely complex formation between DhaQ and DhaS and binding of Dha to DhaQ combined with the finding that DhaS and DhaR are positive regulators of the dha operon and that Dha is the inducer together strongly suggest that DhaS utilizes as the "macromolecular inducer" the Dha-binding protein subunit DhaQ and not a low molecular weight ligand like all other known members of the TetR repressor family (21).

Characterization of the DhaS-DNA Operator Complex—As shown above DhaS belongs to the TetR family of transcription factors, which employ a helix-turn-helix motif for DNA binding (6). Electrophoretic mobility shift assays confirmed binding of DhaS without a histidine tag to the intergenic binding (6). Electrophoretic mobility shift assays confirmed binding of DhaS without a histidine tag to the intergenic region between the dhaS and dhaK genes (Fig. 5C). The minimal length DNA fragment recognized by DhaS extends from bp −54 to bp −79 with respect to the start codon of the dhaKLM operon. DhaS did not bind to unrelated sequences (for instance to the promoter operator of the E. coli dha operon, results not shown) indicating that the binding reaction is specific. The DhaQ subunit added together with DhaS did, however, produce a supershift indicating that the DhaS-DhaQ complex is not stable in the electrophoretic mobility shift assay.

The dha promoter/operator region was further characterized by primer extension analysis and DNase I footprinting. The transcription start point of the dha operon was located to the adenine nucleotide at 22-bp upstream of the dhaKLM start codon (Fig. 6A). The region that is protected by DhaS without a histidine tag was identified by DNase I footprinting. It comprises 28 bp centered at 67-bp upstream of the dhaKLM start codon and 47-bp upstream of the transcription start nucleotide (Fig. 6B). Adding DhaQ with and without Dha to the incubation suggested that the two subunits might form a complex. And indeed, a stable complex between DhaS and DhaQ could be isolated when two cytoplasmic extracts containing DhaQ with a histidine tag and DhaS without a histidine tag, respectively, were mixed and purified by Ni2+ -NTA chromatography (Fig. 5A, inset). The DhaS-DhaQ complex was then separated from excess DhaQ by gel filtration on a Superdex-75 column (Fig. 5A) and its subunit stoichiometry estimated to be 1:1 from the staining intensities of the Coomassie Blue-stained protein bands on the polyacrylamide gel (Fig. 5A, inset).
must either rotate away from the core domain, or the target DNA must bend. A peak of DNA curvature is predicted for the proximal repeat and a minimum of curvature for the −10 promoter sequence by BEND, a program to calculate the macroscopic curvature of DNA (27). This DhaS-protected sequence is strongly conserved in four out of six dhaKLM-dhaS intergenic regions from dha operons encoding non-redundant DhaS, DhaQ and Dha kinase subunits (<90% pairwise sequence identity). All four are of lactococcal and streptococcal origin suggesting a common mechanism of dha operon control. The remaining two putative promoter regions of Bacillus halodurans and Bacillus cereus, are similar to each other but unrelated to the lactococcal sequences (not shown).

The DhaQ-DhaS Binding Interface—A comparison of the conserved amino acid sequence patterns of six non-redundant DhaQs with the corresponding six DhaKs was used to identify the putative protein-protein binding surface. DhaQ and DhaK interact with different protein subunits; DhaQ with DhaS, and DhaK with the catalytic phosphotransferase subunit DhaL (Fig. 1A). Assuming that functionally important interfaces are better conserved than the average surface, the amino acid sequences of the DhaQ and DhaK subunits were aligned separately (supplemental Fig. S1). The similarity patterns were then projected onto the experimental DhaQ structure for comparison of the structures by using the program CONSURF (28) (Fig. 3, F and G). In DhaQ helix 7 is strongly conserved mainly caused by the conservation of five surface exposed, α-helically phased residues, which are not conserved in DhaK. We therefore propose that helix 7 participates in DhaS binding and that the conformational change triggered by Dha binding to His-215 is propagated through the cantilever to helix 7 and hence to DhaS. A second difference is seen between the surfaces around the Dha binding sites (yellow), which is better conserved in the catalytic DhaK subunit than in DhaQ. It is to this area that the DhaL-ATP subunit must dock for the transfer of phosphate from ATP to Dha (2). Overall, the amino acid sequences of the N-terminal α/β fold (residues 1–182) is better conserved in the DhaKs (61% identity) than in the DhaQs (44% identity). For the C-terminal domain the difference of conservation is smaller, namely 56% for DhaK and 47% for DhaQ.
Tracing the function of DhaQ must not be confused with the function of TetR, which is different. It is an activator of transcription that is autoinducers (21). DhaS which undoubtedly also belongs to this family, is different. It is an activator of transcription that is activated by a protein ligand, DhaQ. Like many other transcription activators, DhaS binds to an operator sequence upstream of and only partially overlapping with the −35 promoter consensus sequence (29), whereas the QacR and TetR repressors bind to inverted repeats overlapping with or downstream of the −10 region of the promoter (30, 31). Parenthetically, the activating function of DhaQ must not be confused with the function of “reverse” TetR mutants that bind DNA only in the presence of a ligand (32), unlike wild-type TetR that dissociates from DNA in the absence of the inducer. Electrophoretic mobility shift as well as DNase footprinting indicate that binding of DhaS to the operator DNA is not influenced by the coactivator DhaQ and the inducer Dha. Similar behavior has been observed with PobR the transcription activator for p-hydroxybenzoate hydroxylase (33). The DhaQ-Dha complex may induce a conformational change in DhaS, leading to enhanced RNA polymerase binding. It is not known, whether the DhaS-DhaQ complex activates transcription by distorting the DNA structure or by allosteric interaction with the sigma RNA polymerase.

A BLAST search of the microbial genomes for DhaS homologs produced eleven dhAS genes that were genetically linked to dhAQ genes and operons for Dha kinases. All of them occur in genomes from Bacillales and Lactobacillales. The six DhaSs with non-redundant amino acid sequences (<90% sequence identity; Swiss-Prot IDs Q9CIV9, 8P228, Q8E3R3, Q9K7G7, Q73CL2, Q88Z8X) display pairwise sequence identities between 22 and 45% over the full-length. The identities are between 35 and 65% for the DNA binding domain (α1-α3) and helix α4, between 25 and 30% for the kinked helix 5 (α5) whereas the dimerization helixes α6-α8 are not conserved. The second most closely related group of DhaS homologs (for instance Q8Y9P4 and Q893H0) is encoded with genes associated with putative myosin-cross-reactive antigen, short chain oxidoreductases, and hypothetical proteins. One representative with an open structure like DhaS is TM1030 of Thermotoga maritima (Fig. 2C). However, none of these proteins has been functionally characterized and it is thus not known, whether they are activators like DhaS or repressors like TetR.

In conclusion, control of the dha operon in L. lactis and E. coli occur by different mechanisms. In E. coli, the DhaK and DhaL subunits have a dual function. They catalyze phosphorylation of Dha and at the same time act as mutually antagonistic corepressor and coactivator of an enhancer binding protein (8). In L. lactis, DhaQ, a paralog of the catalytic DhaK subunit, acts as the inducer binding coactivator of a transcription activator from the TetR family. L. lactis and E. coli respond to the same inducer, Dha, but they employ different mechanisms to regulate dha gene expression.
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