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Comparative Genomic Analysis of *dha* Regulon and Related Genes for Anaerobic Glycerol Metabolism in Bacteria

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The dihydroxyacetone (*dha*) regulon of bacteria encodes genes for the anaerobic metabolism of glycerol. In this work, genomic data are used to analyze and compare the *dha* regulon and related genes in different organisms in silico with respect to gene organization, sequence similarity, and possible functions. Database searches showed that among the organisms, the genomes of which have been sequenced so far, only two, i.e., *Klebsiella pneumoniae* MGH 78578 and *Clostridium perfringens* contain a complete *dha* regulon bearing all known enzymes. The components and their organization in the *dha* regulon of these two organisms differ considerably from each other and also from the previously partially sequenced *dha* regulons in *Citrobacter freundii*, *Clostridium pasteurianum*, and *Clostridium butyricum*. Unlike all of the other organisms, genes for the oxidative and reductive pathways of anaerobic glycerol metabolism in *C. perfringens* are located in two separate organization units on the chromosome. Comparisons of deduced protein sequences of genes with similar functions showed that the *dha* regulon components in *K. pneumoniae* and *C. freundii* have high similarities (80−95%) but lower similarities to those of the *Clostridium* species (30−80%). Interestingly, the protein sequence similarities among the *dha* genes of the *Clostridium* species are in many cases even lower than those between the *Clostridium* species and *K. pneumoniae* or *C. freundii*, suggesting two different types of *dha* regulon in the *Clostridium* species studied. The in silico reconstruction and comparison of *dha* regulons revealed several new genes in the microorganisms studied. In particular, a novel *dha* kinase that is phosphoenolpyruvate-dependent is identified and experimentally confirmed for *K. pneumoniae* in addition to the known ATP-dependent *dha* kinase. This finding gives new insights into the regulation of glycerol metabolism in *K. pneumoniae* and explains some hitherto not well understood experimental observations.

Introduction

The anaerobic metabolism of glycerol has been the objective of intensive research in the 1970s and 1980s primarily because of some peculiar biochemical properties of one of the key enzymes, glycerol dehydratase, e.g., coenzyme B_{12} dependency and inactivation by its substrate (for reviews see refs 23 and 25). Recently, anaerobic metabolism of glycerol has received renewed interest because of the industrial utilization of the product 1,3-propanediol as an important monomer for a novel copolymer polytrimethylene terephthalate (PTT) (*6*, *9*). Although 1,3-propanediol is presently mainly produced by a chemical route, there is a worldwide interest in producing this chemical by a biotechnological route, notably demonstrated by the efforts of leading companies such as DuPont (*17*) and Genencor (*10*).

Biologically, 1,3-propanediol can be produced by anaerobic fermentation of glycerol. The metabolic pathways for the anaerobic metabolism of glycerol and formation of 1,3-propanediol are summarized in Figure 1. The anaerobic metabolism of glycerol is a dismutation process. It involves a reductive pathway to convert glycerol first to 3-hydroxypropionaldehyde by the enzyme glycerol dehydratase (GDHt); this intermediate is then reduced to 1,3-propanediol by the enzyme 1,3-propanediol oxidoreductase (PDOR) under the consumption of reducing power NADH. In a coupled oxidative pathway glycerol is oxidized to dihydroxyacetone by the enzyme glycerol dehydrogenase (GDH). This reaction generates the necessary reducing power for the reductive pathway. In *Klebsiella pneumoniae*, the most widely studied organism

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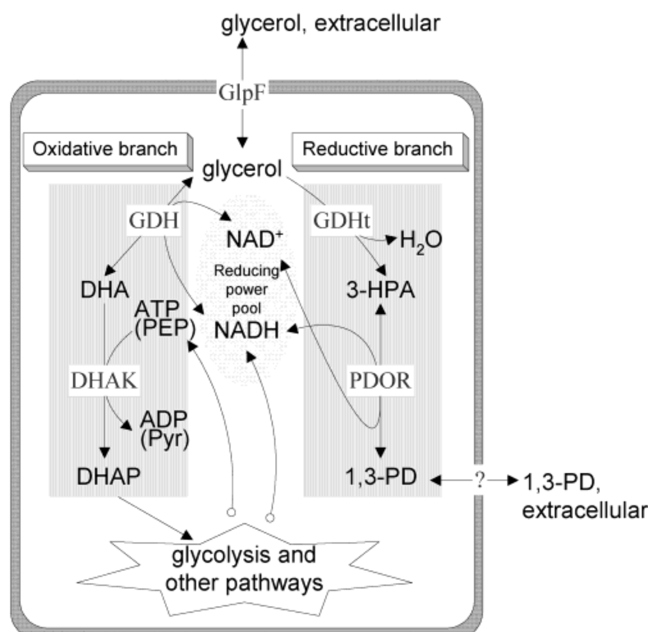


Figure 1. Anaerobic metabolism of glycerol through enzymes encoded by the dihydroxyacetone (dha) regulon in Enterobacteriaceae. DHA: dihydroxyacetone, DHAP: dihydroxyacetone phosphate, 3-HPA: 3-hydroxypropionaldehyde, 1,3-PD: 1,3-propanediol, PEP: phosphoenolpyruvate, Pyr: pyruvate, GlpF: glycerol transport facilitator, GDH: glycerol dehydrogenase, DHAK: dihydroxyacetone kinase, GDHt: glycerol dehydratase, PDOR: 1,3-propanediol oxidoreductase

for glycerol metabolism (*6*), dihydroxyacetone is demonstrated to be phosphorylated through the enzyme dihydroxyacetone kinase (DHAK) and channeled into glycolysis. Forage and Lin (*18*) showed that the four enzymes GDHt, PDOR, GDH, and DHAK are coordinately expressed and the expression is induced by dihydroxyacetone (*dha*) or glycerol. Tong et al. (*50*) found that in *K. pneumoniae* the genes for these four enzymes are organized as a cluster on the chromosome. This genetic unit is therefore named the *dha* regulon. Parts of the *dha* regulon were sequenced in *K. pneumoniae* (*45*), *Citrobacter freundii* (*13*, *14*, *42*, *43*), *Clostridium pasteurianum* (*28*, *29*), and recently also in *Clostridium butyricum* (*41*).

From the sequenced parts of the *dha* regulon, several authors have tried to use a genetic approach (metabolic engineering) to optimize the production of 1,3-propanediol from glycerol (*32*, *46*, *49*, *50*) and glucose (*9*, *16*, *17*). Impressive progresses have been made to produce 1,3-propanediol from glycerol or glucose from transformed *E. coli* that originally does not contain the *dha* regulon. However, metabolic engineering of organisms that contain a native *dha* regulon often did not result in significant improvement of propanediol formation. For example, Menzel (*32*) overexpressed GDHt and PDOR in *K. pneumoniae*. However, the recombinant strain did not produce more 1,3-propanediol than the wild-type strain under real fermentation conditions. Zeng (*53*) postulated a strong feedback inhibition and global regulations of gene expression of the *dha* regulon. The exact genetic basis and mechanisms for these kinds of feedback inhibition and global regulation are not yet clear.

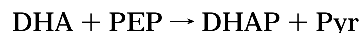
Recently, the whole genome of a *K. pneumoniae* strain MGH 78578 was sequenced and published as contigs (http://genome.wustl.edu). To gain more insights into the genetic organization and regulation of genes of the *dha* regulon we reconstructed a larger and more complete *dha* regulon for *K. pneumoniae* from the genome data in this work. Database searches were also made to find and reconstruct the *dha* regulon in other organisms in order to compare the location, sequence similarity, and functions of *dha* regulon genes in different organisms. This kind of information may be useful for a rational metabolic engineering of the glycerol metabolism for microbial production of 1,3-propanediol.

Materials and Methods

Strains and Cultivation. Three different strains, *K. pneumoniae* DSM 2026, *C. pasteurianum* DSM 525, and *C. butyricum* VPI 1718, were used for experimental studies. *K. pneumoniae* DSM 2026 and *C. pasteurianum* DSM 525 were obtained from the German Collection of Microorganisms (DSMZ), and *C. butyricum* VPI 1718 from H. Biebl (GBF, Germany). The strains were kept at −70 °C and first activated by overnight cultivation in preculture medium as described previously (*31*). *K. pneumoniae* was cultivated aerobically, whereas *C. pasteurianum* and *C. butyricum* were cultivated anaerobically. Five milliliters of the precultures was inoculated into 250-mL anaerobic bottles containing 50 mL of the same preculture medium and cultivated at 37 °C in an incubator.

Enzyme Assays. Cell-free extract was prepared as described by Ahrens et al. (*2*). Assays of the activities of glycerol dehydrogenase, glycerol dehydratase, and 1,3-propanediol oxidoreductase were done as described previously (*2*). The activities of the ATP-dependent dha kinase (DHAK I) and pyruvate kinase were analyzed according to Johnson (*22*) and Ozaki (*36*), respectively. Protein concentration was measured according to Lowry's method (*27*).

The activity of the PEP-dependent dha kinase (DHAK II) was followed at 30 °C through the oxidation of NADH in a coupled reaction system in which the product of DHAK II, dihydroxyacetone phosphate (DHAP), was reduced to glycerol-3-phosphate (G3P) by G3P dehydrogenase (GPDH):



$$\mathrm{DHA + PEP \rightarrow DHAP + Pyr}$$

$$\mathrm{DHAP + NADH \rightarrow G3P + NAD}$$

The reaction mixture contained 1.11 mM DHA, 2.78 mM $MgCl_2$, 0.56 mM NADH, 2.78 mM DTT, 11.1 mM 2,2′-dipyridyl, and 55.6 mM KPi, pH 7.5, in 900 μL volume. After adding 1−50 μL of cell free extract, the optical density (OD) was followed at 340 nm for 2 min. Then, 100 μL of 10 mM PEP and 2 μg of rabbit GPDH (Roche) were added and OD_{340nm} was followed for another 5 min. DHAK II activity was calculated from the difference of NADH consumption rates before and after the addition of PEP and GPDH. The possible interference by glycerol dehydrogenase is avoided by inhibiting this enzyme with the addition of 2,2′-dipyridyl (*22*, *26*).

Reconstruction and Analysis of *dha* Regulon in Different Microorganisms. *K. pneumoniae*. The genome of the *K. pneumoniae* strain MGH 78578 was sequenced by Washington University (http://genome.wustl.edu) recently. A whole genome shotgun approach was used to generate the 7.9 × coverage data given as 398 contigs (state of October 2001). These contigs were downloaded and formatted as a local database of BLAST (Basic Local Alignment Search Tool) (*3*). The genome of the *K. pneumoniae* strain 93 19097 was also sequenced but is only commercially available (*PathoGenome Database*, Genome Therapeutics Corporation, http://www.genomecorp.com). It was searched through LabOnWeb (https://www.labonweb.com).

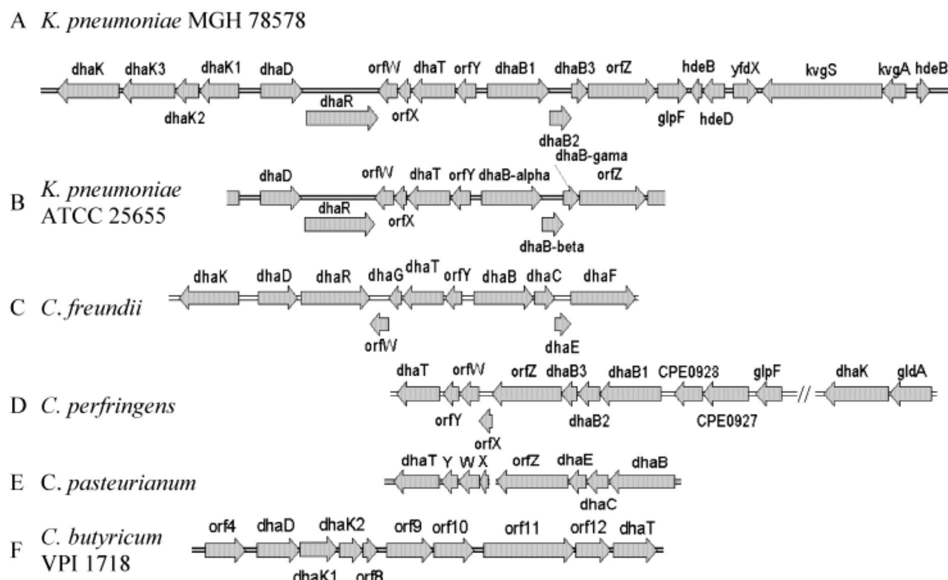


Figure 2. *dha* regulon and related genes in different organisms. (A) *Klebsiella pneumoniae* MGH 78578. (B) *K. pneumoniae* ATCC 25655. (C) *C. freundii*. (D) *C.* perfringens (the orfs of *dhaT*, *orfY*, *orfW*, *orfX*, *orfZ*, *dhaB3*, *dhaB2*, *dhaB1*, *dhaK* are renamed from *CPE00936*, *CPE00935*, *CPE00934*, *CPE00933*, *CPE00932*, *CPE00931*, *CPE00930*, *CPE00929,* and *CPE0100*, respectively; The product of *CPE0928* and *CPE0927* are annotated as regulator (TcsA) and sensor (TcsS) proteins of a two-component signal transduction system). (E) C. *pasteurianum*. (F) *C. butyricum* VPI 1718.

By BLAST searching the genome database of *K. pneumoniae* MGH 78578, two contigs (contig387 and 230) were found to have strong similarities to the previously published partial *dha* regulon sequence of *K. pneumoniae* ATCC 25655 (Genbank AX085388). Using Vector NTI (InforMax, USA) these two contigs can be assembled on the basis of the overlapping area between them. Gaps inside the contigs were closed by insertion of consensus sequences based on comparison between protein sequences deduced from the predicted open reading frames (ORFs) and protein sequences in public protein databases (PIR and GenPept), which have similar function. Because gaps shift transcription frames, the gap correction may affect the comparison between translated proteins. The ORFs of the assembled fragment were analyzed by using Vector NTI and compared again to public databases. Web service of INTERPRO (http://www.ebi.ac.uk/interpro) (*4*) was used to find motifs and to predict the function of new identified sequences.

Since there are genes of the *dha* regulon lying on the left end of the reassembled large fragment, it was of interest to examine whether there are other genes in the neighborhood that are also related to the *dha* regulon. With the help of a small piece of DNA sequence from the *PathoGenome Database*, another contig (contig226) was found to relate to the *dha* regulon. Hence, an even bigger fragment of DNA could be assembled that bears the *dha* regulon and related genes of *K. pneumoniae* (Figure 2A). The sources of the DNA sequences are given in Table 1.

Clostridium perfringens. *C. perfringens* is a Gram-positive anaerobic spore-forming bacterium that causes life-threatening gas gangrene and mild enterotoxemia in humans. Recently, the genome of *C. perfringens* strain 13 is completely sequenced and partially annotated (Genome: GenBank NC003366) (*44*). By searching this genome through website of NCBI (http://www.ncbi.nlm.nih.gov/BLAST), a complete putative *dha* regulon was found (Figure 2D, Table 1). The names of some of the ORFs are assigned on the basis of sequence similarity to genes in the databases.

Organisms with Partial Sequences of *dha* Regulon. The maps of *dha* regulon in *K. pneumoniae* ATCC 25655 (Figure 2B), *C. freundii* DSM 30040 (Figure 2C), and *C. pasteurianum* DSM 525 (Figure 2E) are constructed on the basis of the corresponding entries in Genbank (Table 1). Some of the ORFs are renamed according to literature (*12*, *17*, *42*). For example, the DNA entry for the *dha* regulon of *K. pneumoniae* ATCC 25655 (GenBank U30903) named orf 3c as dhaB4 and its product as the large subunit of glycerol dehydratase. In fact, its product should be the large subunit of glycerol dehydratase activator. We reassigned it as orfZ according to literature (*17*).

Clostridium butyricum VPI 1718 has a coenzyme B_{12} independent glycerol dehydratase (*40*, *41*). The *dha* regulon in this organism is constructed on the basis of Genbank sequences (Genbank AX074561, AX074557, AX074558, AX074560) and unpublished results of P. Soucaille (Figure 2F).

Results and Discussion

Database searches showed that among the organisms the genomes of which have been sequenced (state of 1st February 2002), only two organisms, i.e., *K. pneumoniae* MGH 78578 and *C. perfringens* contain a complete *dha* regulon. Beside the previously known genes of the partial *dha* regulon in *K. pneumoniae* ATCC 25655 (Figure 2B), the reassembled *dha* regulon of *K. pneumoniae* MGH 78578 also encodes several new genes related to the *dha* regulon (Figure 2A). The ORFs *dhaK*, *dhaK1*, *dhaK2*, and *dhaK3* are newly identified genes. *DhaK* encodes a known dihydroxyacetone kinase (DHAK I) of *K. pneumonia* that is ATP-dependent (*39*). *DhaK1*, *dhaK2*, and *dhaK3* have high identities to the genes *dhaK1* (*ycgT)*, *dhaK2* (*ycgS*), and *dhaH* (*ycgC*) of *E. coli*, respectively (Table 4), which encode the three soluble protein subunits of a dihydroxyacetone kinase (DHAK II) that uses phosphoenolpyruvate (PEP) instead of ATP as phosphoryl donor (*20*). In the work of Gutknecht et al. (*20*) *dhaK1* (*ycgT*), *dhaK2* (*ycgS*), and *dhaH* (*ycgC*) were named as *dhaK*, *dhaL*, and *dhaM*, respectively. In the right-hand half of the DNA fragment (Figure 2A) a gene (*glpF*) encoding a glycerol transport facilitator and several other genes related to global regulation (*hdeBD*) and genes

Table 1. Sources of DNA Sequences Used in This Work

strain	sequence ID	sequence length (base pairs)	source
Klebsiella pneumoniae MGH 78578	contig387	53,721	Washington University
	contig230	4,969	Washington University
	contig226	5,968	Washington University
Klebsiella pneumoniae strain 93 19097	7504987761	1,747	PathoGenome Database
Klebsiella pneumoniae ATCC 25655	U30903	7,926	GenBank
Klebsiella pneumoniae ATCC 25655	AX082621	12,145	GenBank
Clostridium perfringens	NC_003366 (1135200-1124400)	10,801	GenBank
	NC_003366 (139300-136000)	3,301	GenBank
Citrobacter freudii	U09771	13,128	GenBank
Clostridium pasteurianum	AF006034	2,881	GenBank
	AF051373	4,640	GenBank
Clostridium butyricum		12,500	P. Soucaille

encoding a two-component signal transduction system (*kvgA* and *kvgS*) are also identified. The major components of the *dha* regulons in different microorganisms and their nomenclature as used in the literature are summarized in Table 2. The *dha* regulons are compared to each other with respect to components, organization, sequence similarity, and functionality.

Components and Organization. *dhaB1*, *dhaB2*, *dhaB3*, *orfX*, *orfZ*, *dhaT*, *orfW*, and *orfY* are common components of *dha* regulon in the organisms *K. pneumoniae*, *C. freudii*, *C. pasteurianum,* and *C. perfringens*. Except for *orfW* and *orfY*, the functions of which are unknown, all of them belong to the reductive pathway of glycerol utilization. In both of the sequenced genomes (*K. pneumoniae* and *C. perfringens*), a gene encoding for glycerol transport facilitator (*glpF*) is found in the vicinity of the common *dha* regulon gene. It is not known if *glpF* is coordinately regulated with the *dha* genes and belongs to the *dha* regulon.

K. pneumoniae is unique in that its *dha* regulon contains two types of dha kinases. One of them (DHAK I), similar to the dha kinase in *C. freundii* and *C. perfringens*, has only one encoding gene, whereas the other (DHAK II), similar to the phosphoenolpyruvate-dependent dha kinase in *E. coli* (*20*), has three. *K. pneumoniae* and *C. freundii* have a regulator gene *dhaR* that is not found in other organisms. *C. perfringens* has two different putative regulatory proteins *CPE0928* (sensor of a two-component signal transduction system, termed here as *tcsS*) and *CPE0927* (activator of a two-component signal transduction system, *tcsA*) (*44*) that may function as the regulatory protein of the *dha* regulon (see Figure 2D, not shown in Table 1). Interestingly, the *dha* regulon of *C. butyricum* is considerably different from that in all the other organisms. It has only one ORF (*orf11*) encoding a coenzyme B_{12} independent glycerol dehydratase and only one ORF (*orf12*) encoding its activator. Moreover, *C. butyricum* has also two-component signal transduction proteins, encoded by *orf9* and *orf10*, instead of *dhaR* and a dha kinase that might have three encoding ORFs (*dhaK1*, *dhaK2*, and *orf8*).

Figure 2 shows that the *dha* regulon and related genes in almost all the mentioned organisms are organized together except for *C. perfringens*, in which the *dha* regulon is located in two spatially different clusters. However, in all of the organisms, genes belonging to the reductive pathway and genes belonging to the oxidative pathway are closely organized. The *dha* regulon of the taxonomically closely related *K. pneumoniae* and *C. freundii* are organized similarly (Figure 2). The genes in the *dha* regulon of these organisms are divergently transcribed. On the other hand, the organization of the *dha* regulon genes for the reductive pathway of glycerol metabolism in *C. pasteurianum* and *C. perfringens* is similar to each other but quite different from that of *K. pneumoniae* and *C. freundii*. All of the genes for the reductive pathway of the *dha* regulon in *C. pasteurianum*, *C. butyricum,* and *C. perfringens* are transcripted in the same direction. The genes encoding the activator (*42*) of glycerol dehydratase, namely, *orfZ* and *orfX*, are neighbors in these organisms, whereas they are separated by other genes in *K. pneumoniae* and *C. freundii* and belong to two different operons. The putative *dha* regulon genes for the oxidative pathway of glycerol in *C. perfringens* and *C. butyricum*, *dhaD* and *dhaK*, are also localized in the same DNA strand, whereas these two genes are arranged in opposite strands in *K. pneumoniae* and *C. freundii*. The differences in the components and organization of genes indicate the existence of diverse *dha* regulons in microorganisms. In the following section the sequence similarity and functionality of the *dha* regulons are discussed in more detail.

Sequence Similarities. Both the nucleic acid sequences and deduced protein sequences of *dha* regulon genes in *K. pneumoniae* MGH78578 and *K. pneumoniae* ATCC25655 are highly similar (>98% identity) (Table 3), suggesting that these two strains should behave very much the same with regard to anaerobic metabolism of glycerol. Sequences of proteins encoded by the *dha* regulon genes in *K. pneumoniae* are mostly about 80−90% similar to those of the corresponding proteins in *C. freundii*, while the sequence similarity between the corresponding genes and proteins in *K. pneumoniae* and the three *Clostridium* species is about 10−50% lower. Interestingly, the similarity among the *Clostridium* species is not significantly higher than the similarity between *Clostridium* and *Klebsiella* or *Citrobacter*, indicating heterogeneity of the *dha* regulon in the *Clostridium* species considered. In fact, *C. butyricum* has a very different *dha* regulon compared to that of *C. pasteurianum* and *C. perfringens*. This is in accordance with the experimental observation that *C. butyricum* VPI 1718 behaves phenotypically differently from *C. pasteurianum* in anaerobic fermentation of glycerol (*24*). For example, *C. butyricum* VPI 1718 produces much less hydrogen but achieves a higher yield of 1,3-propanediol than *C. pasteurianum.* Recently, Biebl and Spröer (*7*) conducted a taxonomic and phylogenetic study of glycerol fermenting Clostridia. The 16S rDNA sequences of *C. butyricum* VPI 3266, which according to DNA-DNA hybridization data is very closely related to *C. butyricum* VPI 1718 (*11*), and *C. pasteurianum* were shown to be very different. No experimental work has been reported for *C. perfringens* with respect to glycerol fermentation.

In all of the common components, the large subunit of GDHt (encoded by *dhaB1*) and the enzyme PDOR have similarities higher than 75% between any two organisms considered in this work, indicating that they are highly

Table 2. Major Components, Their Nomenclature, and Size[a] of *dha* Regulons in *Klebsiella pneumoniae* (Kpn), *Citrobacter freundii* (Cfr), *Clostridium pasteurianum* (Cpa), *Clostridium perfringens* (Cpe), and *Clostridium butyricum* VPI 1718 (Cbu)[b]

component protein	complete name	genes encoding the protein in different organisms					
		Kpn MGH78578[c]	Kpn ATCC25655[d]	Cfr[d]	Cpa[d]	Cpe[c]	Cbu[e]
GlpF	glycerol transport facilitator	*glpF* (810)	*glpF-β* (partial) (529)			*glpF* (705)	
GDHt	glycerol dehydratase	*dhaB1* (1665)	*dhaB-α* (1665)	*dhaB* (1665)	*dhaB* (1665)	*dhaB1* (1665)	
		dhaB2 (582)	*dhaB-β* (582)	*dhaC* (582)	*dhaC* (540)	*dhaB2* (573)	
		dhaB3 (429)	*dhaB-γ* (429)	dhaE (432)	*dhaE* (441)	*dhaB3* (426)	
							orf11 (2409)
GDHt activator	GDHt activator	*orfX* (351)	*orfX* (351)	*dhaG* (351)	*orfX* (partial) (207)	*orfX* (351)	
		orfZ (1821)	*orfZ* (1821)	*dhaF* (1809)	*orfZ* (1809)	*orfZ* (1851)	
							orf12 (912)
PDOR	1,3-propanediol oxidoreductase	*dhaT* (1161)	*dhaT* (1161)	*dhaT* (1161)	*dhaT* (1155)	*dhaT* (1158)	*dhaT* (1155)
DhaR	regulatory protein	*dhaR* (1923)	*dhaR* (1923)	*dhaR* (1923)			
						CPE0927 (1233)	*orf9* (1215)
						CPE0928 (753)	*orf10* (1053)
GDH	glycerol dehydrogenase	*dhaD* (1122)	*dhaD* (1122)	*dhaD* (1104)		*gldA* (1128)	*dhaD* (1140)
DHAK	dihydroxyacetone kinase	*dhaK1* (1068)	*dhaK1* (partial) (399)				*dhaK1* (1002)
		dhaK2 (630)					*dhaK2* (627)
		dhaK3 (1419)					*orf8* (384)
		dhaK (1647)		*dhaK* (1656)		*dhaK* (1746)	
other common proteins with unknown functions	OrfW	*orfW* (528)	*orfW* (528)	*orfW* (528)	*orfW* (519)	*orfW* (513)	
	OrfY	*orfY* (546)	*orfY* (546)	*orfY* (492)	*orfY* (429)	*orfY* (429)	

[a] The number of base pairs is given in parentheses. [b] Only common and comparable genes are showed in this table. [c] Gene names are assigned based on sequence similarity to GenBank. [d] Gene names are taken from literature (see text). [e] Gene names are either from literature or based on sequence similarity.

conserved components of the *dha* regulon. The other two subunits of GDHt and one of the activator subunit encoded by *orfZ* (with similarities more than 60%) are also relatively well conserved. The enzymes for the oxidative pathway (GDH and DHAK) are less well conserved (Tables 3 and 4). In other words, the glycerol-fermenting organisms have kept the reductive pathway but not the oxidative pathway conserved in the evolution. This may be due to the fact that the reductive pathway is essential for an organism to grow anaerobically on glycerol when there is no other electron acceptor.

As mentioned above, *K. pneumoniae* has two dha kinases that differ from each other (Table 4). The first one (DHAK I) has only one subunit (DhaK) and is 84.9% identical to the one in *C. freundii*. The second one (DHAK II) has three subunits (DhaK1, DhaK2, and DhaK3) that are 87%, 83%, and 64.4% identical to the three subunits of the dha kinase of *E. coli*, respectively (for more discussion see below). The strain *C. butyricum* VPI 1718 has a putative dha kinase with three coding ORFs that are more similar to the three-ORF dha kinase (DHAK II) than to the one-ORF kinase (DHAK I) of *K. pneumoniae*. The *C. perfringens* dha kinase is encoded by one ORF that is surprisingly also more similar to the three-ORF dha kinase than to the other type.

Functionality of Related Genes. The functions of some of the genes in the *dha* regulon of *K. pneumoniae*, *C. freundii*, and C. *pasteurianum* have been previously investigated and discussed (*12*, *13*, *28*, *29*, *45*, *50*). Briefly, *dhaB* (B1, B2, and B3) and *dhaT* encode the two enzymes of the reductive pathway of glycerol metabolism, namely, glycerol dehydratase (GDHt) and 1,3-propanediol oxidoreductase (PDOR), respectively. *OrfX* and *orfZ* encode an activator of glycerol dehydratase (*42*). *dhaD* and *dhaK* encode the two enzymes of the oxidative pathway, namely, glycerol dehydrogenase (GDH) and dihydroxyacetone kinase (DHAK), respectively. *DhaR* encodes a positive regulatory protein for at least the *dhaT* and *dhaB* genes of the *dha* regulon (*32*). *OrfW* and *orfY* were initially identified in the *dha* regulon of *K. pneumoniae* ATCC 25655; their functions are not clear. In the following the functionality of these not well characterized genes and the new identified related genes are discussed and compared for different organisms.

***dhaR* and Other Regulatory Genes.** The domain structure of the deduced regulator DhaR and other potential regulatory proteins in different organisms was analyzed with the INTERPRO database. DhaR of *K. pneumoniae* and *C. freundii* contains GAF (52−199) (*15*), PAS (203−267) (*48*), and sigma 54 (σ^{54}) factor interaction

Table 3. Comparison of Protein Identity (%) and Nucleic Acid Sequence Identity (in Parentheses) of *dha* Regulon Genes between Different Organisms

gene	organisms	Kpn25655	Cfr	Cpa	Cbu	Cpe
dhaB1	Kpn78578	100 (99.1)	93.7 (83.9)	78.2 (63.2)	10.9 (41.1)[a]	80.9 (63.3)
	Kpn25655		93.7 (83.8)	78.2 (63.3)	10.9 (40.6)[a]	80.9 (63.2)
	Cfr			77.8 (65.1)	12.0 (42.2)[a]	80.4 (65.0)
	Cpa				12.6 (48.9)[a]	79.8 (76.5)
	Cbu					11.6 (49.4)[a]
dhaB2	Kpn78578	99.5 (99.3)	88.7 (79.5)	67.2 (56.6)	14.4 (42.4)[a]	67.5 (57.3)[a]
	Kpn25655		89.2 (79.0)	67.0 (56.8)	14.2 (42.6)[a]	67.0 (57.3)
	Cfr			67.0 (60.1)	10.6 (44.6)[a]	69.6 (61.6)
	Cpa				9.6 (49.4)[a]	70.0 (71.1)
	Cbu					12.3 (49.8)[a]
dhaB3	Kpn78578	100 (100)	84.3 (78.8)	61.3 (53.6)	11.0 (40.6)[a]	61.5 (53.2)
	Kpn25655		84.3 (79.9)	61.3 (54.2)[a]	11.0 (40.9)[a]	61.5 (53.2)
	Cfr			59.9 (54.9)	10.9 (45.2)[a]	61.3 (55.5)
	Cpa				15.0 (51.1)[a]	64.4 (69.7)
	Cbu					13.6 (50.0)[a]
orfZ	Kpn78578	99.8 (99.8)	83.0 (78.0)	61.5 (55.1)	8.6 (40.9)[b]	64.1 (55.5)
	Kpn25655		83.1 (78.1)	62.0 (55.0)	8.6 (40.6)[b]	64.3 (55.4)
	Cfr			61.8 (57.0)	11.0 (43.0)[b]	64.9 (57.7)
	Cpa				11.3 (49.1)[b]	69.5 (71.8)
	Cbu					9.7 (49.4)[b]
orfX	Kpn78578	99.1 (99.7)	62.4 (67.8)	34.4 (40.2)	12.0 (36.3)[b]	33.9 (41.2)
	Kpn25655		63.2 (67.8)	33.9 (40.1)	12.4 (37.0)[b]	33.9 (41.4)
	Cfr			30.3 (39.5)	13.3 (42.8)[b]	33.1 (44.9)
	Cpa				15.9 (53.3)[b]	37.5 (54.3)
	Cbu					13.7 (52.5)[b]
dhaT	Kpn78578	99.7 (99.2)	93.5 (83.5)	79.1 (64.4)	75.7 (62.4)	81.9 (64.3)
	Kpn25655		93.3 (83.2)	78.8 (64.0)	75.5 (62.0)	81.7 (63.9)
	Cfr			79.8 (65.9)	77.0 (63.9)	82.2 (66.6)
	Cpa				85.5 (82.0)	85.7 (78.5)
	Cbu					86.8 (79.7)
dhaD	Kpn78578	99.7 (99.6)	90.6 (83.7)		43.7 (49.7)	43.4 (49.9)
	Kpn25655		90.8 (81.4)		43.5 (49.4)	43.2 (48.7)
	Cfr				45.0 (52.7)	44.7 (50.8)
	Cpa					
	Cbu					74.2 (72.6)
dhaR	Kpn78578	99.8 (99.3)	91.7 (82.5)			
	Kpn25655		91.6 (82.5)			
	Cfr					
	Cpa					
	Cbu					
orfW	Kpn78578	98.9 (98.9)	77.8 (75.7)	46.0 (47.3)		54.0 (51.2)
	Kpn25655		79.0 (76.5)	46.6 (47.6)		54.0 (50.9)
	Cfr			48.9 (51.8)		56.3 (51.8)
	Cpa					56.6 (67.0)
	Cbu					
orfY	Kpn78578	98.4 (98.7)	79.6 (74.6)	53.1 (53.5)		55.6 (56.1)
	Kpn25655		79.4 (73.7)	53.5 (53.4)		54.5 (56.4)
	Cfr			53.1 (58.1)		59.9 (61.5)
	Cpa					65.0 (71.8)
	Cbu					

[a,b] orf11 and orf12 of *Clostridium butyricum* VPI 1718 (see Table 2 and Figure 2) are used for comparison, respectively.

Table 4. Comparison of Identities of Protein Sequence and Nucleic Acid Sequence (in Parentheses) between *dha* Kinases of Selected Microorganisms (%)

	DhaK1_Kpn	DhaK2_Kpn	DhaK3_Kpn	DhaK_Cfr	DhaK1_Eco	DhaK2_Eco	DhaM_Eco	DhaK1_Cbu	DhaK2_Cbu	Orf8_Cbu	DhaK_Cpe
DhaK_Kpn	27.2 (53)	26.9 (48)	13.2 (48)	84.9 (79)	27.2 (49)	26.5 (47)	14.6 (46)	33.6 (46)	27.9 (40)	11.0 (40)	28.7 (43)
DhaK1_Kpn		15.1 (48)	16.4 (47)	25 (51)	87.1 (78)	14.2 (47)	14.3 (48)	42.9 (45)	13.4 (41)	12.1 (44)	41.7 (48)
DhaK2_Kpn			14.0 (48)	25.6 (49)	16.0 (46)	83.0 (77)	12.2 (47)	11.2 (40)	35.2 (45)	13.9 (43)	26.7 (42)
DhaK3_Kpn				15.1 (48)	14.7 (46)	14.1 (47)	64.4 (64.2)	11.0 (40)	12.7 (39)	37.4 (46)	11.4 (40)
DhaK_Cfr					26.3 (50)	22.5 (51)	12.4 (46)	35.3 (46)	26.6 (43)	11.8 (44)	29.7 (44)
DhaK1_Eco						14.6 (46)	13.6 (46)	41.5 (51)	13.1 (43)	11.5 (45)	39.5 (48)
DhaK2_Eco							12.7 (45)	11.6 (45)	36.7 (49)	14.7 (45)	26.0 (47)
DhaM_Eco								12.8 (45)	12.2 (46)	38.8 (50)	13.3 (45)
DhaK1_Cbu									10.0 (49)	16.2 (52)	52.7 (65)
DhaK2_Cbu										16.8 (50)	32.9 (54)
Orf8_Cbu											13.2 (50)

domains (327–544) (*8*) and a histidine HTH_8 domain (588–628) (*5*). The GAF domain is present in phytochromes and cGMP-specific phosphodiesterases. PAS domains are used in many two-component sensor proteins to sense the internal levels of oxygen, redox potential, energy charge, and light (*48*). HTH-8 domain has a helix-turn-helix conformation and acts as a DNA binding domain (*5*). The regulatory proteins containing a σ^{54} factor interaction domain have ATPase activity and can interact with the core RNA polymerase subunit

associated with the σ^{54} factor and activate the RNA transcription from σ^{54} promoters (*8*). On the basis of its domain structure, DhaR of *K. pneumoniae* and *C. freundii* can potentially both sense the intracellular level of important physiological parameters such as redox potential or energy charge and act as an effector for triggering transcription of *dha* regulon genes.

Instead of a DhaR-like regulator, *C. perfringens* and *C. butyricum* VPI 1718 have another putative regulatory system in their *dha* regulon: a two-component signal transduction system consisting of a sensor component and a regulatory component. These components are named CPE0927 (termed here as TcsS) and CPE0928 (termed here as TcsA) in *C. perfringens*, and are named as Orf9 and Orf10 in *C. butyricum*. The 116-amino acid N-termini of the sensor components TcsS and Orf9 have a signal sensing domain and are 58.6% identical, whereas the C-termini of these two sensor proteins have relatively lower similarity (39.3% identical) but still share the same histidine kinase motif. The response regulatory proteins of *C. perfringens* and *C. butyricum* (TcsA and Orf10) also have similar motif structure: the N-terminus receiver domain with 29.2% identities and the C-terminus AraC-like helix-turn-helix motif (HTH-Arac) with 46.1% identities to each other. On the basis of the complete domain structure, it is proposed that the regulation by TcsS/Orf9 and TcsA/Orf10 takes place by the common two-component signal transduction mechanism. This means that the sensor protein of the two-component system senses the environmental signal and transfers such information to the regulator component through phosphorylation, and the latter then initiates RNA transcription (*47*).

Near the *dha* regulon of *K. pneumoniae* MGH 78578, two ORFs *kvgS* and *kvgA* are found to encode two proteins comprising a two-component signal transduction system (Figure 2A) that are 34% and 45% identical to KvgS (GenPept: CAB61240) and KvgA (GenPept: CAB61239) of another clinical isolation of *K. pneumoniae*, respectively. However, KvgS and KvgA of *K. pneumoniae* MGH 78578 have relatively low similarity (<12% identities for KvgS and <18% identities for KvgA) to the two-component signal transduction system TcsS/TcsA of *C. perfringens* and Orf9/Orf10 of *C. butyricum.* In view of the possibility that the two-component signal transduction system in *C. perfringens* and *C. butyricum* may have a role similar to that of DhaR in *K. pneumoniae*, it is interesting to ask the question whether the two-component signal transduction system KvgS/KvgA of *K. pneumoniae* can also contribute to the regulation of the *dha* regulon. In this connection, it is worth mentioning that next to the *glpF* gene in the *dha* regulon of *K. pneumoniae*, there are also several global regulatory genes (*hdeD*, *hdeB*, and *pfdX*) with unknown functions (Figure 2A). Little information is available in this regard.

Glycerol Dehydratase and Its Activator (dhaB1, dhaB2, dhaB3, orfX, and orfZ). Glycerol dehydratase catalyses the first step of the reductive catabolism of glycerol and is considered to be a limiting step of 1,3-propanediol production in *C. butyricum* (*1*) and *K. pneumoniae* (*2*). Glycerol dehydratases of *K. pneumoniae*, *C. freundii*, *C. pasteurianum,* and *C. perfringens* have a subunit composition of $\alpha 2\beta 2\gamma 2$ and contain coenzyme B_{12} in the reaction center like other diol dehydratases (*12*). The three different kinds of subunits are encoded by the genes *dhaB1*, *dhaB2*, and *dhaB3*, respectively. Glycerol dehydratase catalyses the reaction via a radical mechanism. It is subject to inactivation by its natural substrate glycerol. More and more experimental evidence suggests that the gene products of *orfX* and *orfZ* can both reactivate the glycerol-deactivated or oxygen-deactivated glycerol dehydratase and protect the dehydratase from deactivation by glycerol effectively in the presence of coenzyme B_{12}, ATP, and Mg^{2+}, thus functioning as an activator of the glycerol dehydratase (*33*–*35*, *42*, *51*, *52*).

In contrast to the well-known B_{12}-dependent glycerol dehydratase in other organisms *C. butyricum* VPI 1718 has a B_{12}-independent glycerol dehydratase (*41*). Orf11p and Orf12p were confirmed to work as a B_{12}-independent glycerol dehydratase through transformation experiments (*41*). Interestingly, their sequences seem similar to the pyruvate-formate lyase (PFL) (22% identities) and the PFL activator (29% identities) of *E. coli*. *Escherichia coli* is known to have no native glycerol or diol dehydratase and achieved the ability to convert glycerol to 1,3-propanediol when it was transformed with these two genes from *C. butyricum* VPI 1718. This proves that these two genes encode a new type of glycerol dehydratase and activator. Although this new glycerol dehydratase does not need coenzyme B_{12}, it depends on *S*-adenosyl-methionine (SAM) as a cofactor (P. Soucaille et al., submitted for publication).

Glycerol Transport Facilitator GlpF. With an identity of 80.9% the GlpF protein of *K. pneumoniae* is very similar to the glycerol transport facilitator of *E. coli*. GlpF is a transmembrane protein belonging to the major intrinsic protein (MIP) superfamily (*37*) and involved in forming aqueous pores that selectively allow passive transport of the solute(s) such as glycerol, dihydroxyacetone, urea, and other small neutral linear molecules (but neither water nor ions) across the membrane (*21*). Since glycerol can pass the membrane by diffusion, the function of glycerol transport facilitator may be significant only when the concentration of glycerol is very low (i.e., lower than 8,mM) (*38*). The crystal structure of the glycerol facilitator of *E. coli* has been solved to 2.2 Å resolution (*19*). It was demonstrated that two successive hydroxyl groups are important to meet the internal structure of the porin. 1,3-Propanediol, therefore, may not be the preferred substrate for GlpF. Because of its higher hydrophobicity compared to that of glycerol, 1,3-propanediol is expected to be transported through the membrane more easily. Till now no active export mechanism has been known for 1,3-propanediol. Therefore, the intracellular concentration of 1,3-propanediol would unduly increase during the production of 1,3-propanediol. Because PDOR catalyses a reversible reaction, the intracellular concentration of 3-HPA can also be very high, leading to killing of the cells if there is no efficient mechanism to remove it.

Novel Dihydroxyacetone Kinase. The DHA kinase type I in *K. pneumoniae* was purified 20 years ago (*22*). It consumes ATP and is highly specific for DHA but not for glycerol. In contrast, glycerol kinase that is expressed aerobically can take either glycerol or DHA as substrate. The ATP-dependent DHA kinase was identified as a homodimer of two 53 KDa subunits (*22*). This is consistent with its predicted size derived from the DNA sequence (57,349 Da).

As mentioned before, a second type of dha kinase (DHAK II) was found in the *dha* regulon of *K. pneumoniae*; it is encoded by three ORFs and is very similar to the newly identified dha kinase in *E. coli* (*20*). The latter consists of three soluble protein subunits; two of them have homogeneity to *dhaK* of *C. freundii*, the third, termed as *dhaM*, has a multidomain structure. The N-terminal dimerization domain has the same folding as the IIA domain of the mannose transporter of the bacterial phosphoenolpyruvate/sugar phosphotransferase

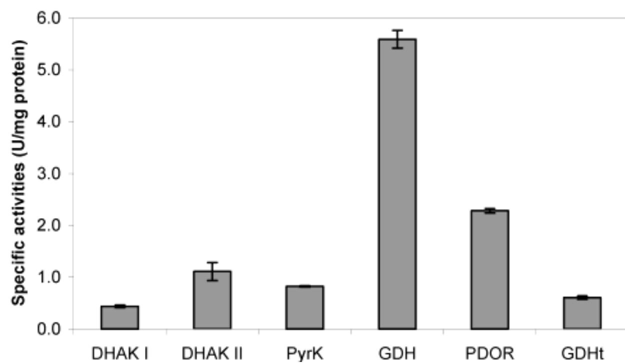


Figure 3. Enzyme activities in anaerobic glycerol fermentation by *K. pneumoniae*.

system (PTS). The middle domain of *dhaM* is similar to HPr, and the C-terminus is similar to the N-terminal domain of enzyme I (EI) of the PTS. Instead of ATP, this kinase uses PEP as a phosphoryl donor, and the PTS system components, EI and HPr, are also important for the catalysis. Since the sequence, domain structure, and conserved active phosphorylation sites of the second DHA kinase of *K. pneumoniae* are all similar to those of DHA kinase of *E. coli*, it is very much likely that they act by similar catalyzing mechanism and have similar functions.

Similarly to *K. pneumoniae*, *C. butyricum* also has a DHA kinase with three subunits. Two of them are similar to the DhaK1 and DhaK2 of *K. pneumoniae*, respectively. The third one (Orf8) (128 amino acids) is much smaller than DhaK3 (473 amino acids) and is only 38.8% identical to the 118 amino acids of N-terminus of DhaK3.

To examine the functionality of DHAK II in different organisms assays of DHAK I and DHAK II activities were carried out with shake flask cultures of *K. pneumoniae* DSM 2026, *C. pasteurianum* DSM 525, and *C. butyricum* VPI 1718 anaerobically grown on glycerol. A specific DHAK II activity of 1.11 U/mg protein was determined for *K. pneumoniae*, which was 2.5 times as high as its activity of DHAK I (0.43 U/mg protein). A significant activity (0.12 U/mg protein) of DHAK II was also found in *C. pasteurianum,* which was, however, much lower than the activity of DHAK I (0.77 U/mg protein) under the experimental conditions. No activity of DHAK II was found for *C. butyricum*. The activity of DHAK II in *K. pneumoniae* was compared with several other key enzymes of glycerol metabolism in Figure 3. The activity of DHAK II was higher than the activities of pyruvate kinase and glycerol dehydratase but lower than the activities of glycerol dehydrogenase and 1,3-propanediol oxidoreductase. The results shown in Figure 3 are consistent with previous observations concerning anaerobic glycerol metabolism in this organism. Ruch and Lin (*39*) and Sprenger et al. (*46*) showed that the activity of the ATP-dependent DHA kinase in *K. pneumoniae* is unusually lower than the activities of GDH and PDOR by a factor greater than 10. Considering the fact that the metabolic fluxes through the enzymes of the oxidative and reductive pathways of glycerol metabolism should be in the same order due to the necessary balance of reducing power as shown in Figure 1, the reason for the observed large difference in the enzyme activities was not clear. It is now obvious that the PEP-dependent DHA kinase significantly contributes to the conversion of DHA to DHAP.

In previous work with glycerol metabolism of *K. pneumoniae* it was also observed that pyruvate kinase (PK) for the conversion of PEP to pyruvate has a quite low activity compared to the enzymes of oxidative glycerol dissimilation (e.g., GDH) and pyruvate metabolism (e.g., pyruvate: formate-lyase) (*2*, *30*). This led to the conclusion that pyruvate kinase is a limiting step in the oxidative pathway of glycerol utilization. Surprisingly, the measured in vitro activity of PK is in some cases even lower than the in vivo activity of this enzyme calculated from the flux of PEP to pyruvate, resulting in a unrealistic ratio of in vitro activity/in vivo activity <1.0 (*2*). These seemingly unrealistic results can now be understood if we consider the contribution of the PEP-dependent DHAK to the conversion of PEP to pyruvate (see Figures 1 and 3).

The above examples demonstrate that genomic information can be very useful for understanding cellular metabolism. More importantly, genomic information about the genetic makeup and regulation of genetic units in different organisms, as discussed in this work for the *dha* regulon, can guide the selection of the optimal organism and the desired genetic modification of metabolic pathways for developing efficient bioprocesses.

Conclusions

The use of genomic data led to the reconstruction of two types of *dha* regulons in *K. pneumoniae* and *C. perfringens* with different elements and organizations. Several new genes and regulatory domains were identified that have not been studied so far for the metabolism of glycerol. In particular, a novel phosphoenolpyruvate-dependent dha kinase encoded by three ORFs was identified and its functionality was confirmed for *K. pneumoniae* in addition to the previously known ATP-dependent DHA kinase. The presence of this new DHA kinase explains several experimental observations concerning glycerol utilization in this organism.

The nucleic acid and protein sequences of *dha* regulon and related genes in the other three organisms (*C. freundii*, *C. pasteurianum*, and *C. butyricum*) were also compared to those of genes having similar functions in *K. pneumoniae* and *C. perfringens*. *dha* regulon genes in the *Klebsiella* and *Citrobacter* species have high similarity (80−95%), whereas they exhibit lower similarity to those of the *Clostridium* species (30−80%). Interestingly, the nucleic acid and protein sequence similarities among the *dha* genes of the *Clostridium* species are in many cases lower than those between the *Clostridium* species and the *Klebsiella* and *Citrobacter* species, revealing two different kinds of *dha* regulons in the *Clostridium* species.

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