A novel adaptation of aldolase regulates virulence in *Streptococcus pyogenes*

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Regulation of virulence factor expression is critical for pathogenic microorganisms that must sense and adapt to a dynamic host environment; yet, the signal transduction pathways that enable this process are generally poorly understood. Here, we identify LacD.1 as a global regulator of virulence factor expression in the versatile human pathogen, *Streptococcus pyogenes*. LacD.1 is derived from a class I tagatose-1,6-bisphosphate aldolase homologous to those involved in lactose and galactose metabolism in related prokaryotes. However, regulation of transcription by LacD.1 is not dependent on this enzymatic activity or the canonical catabolite repression pathway, but likely does require substrate recognition. Our results suggest that LacD.1 has been adapted as a metabolic sensor, and raise the possibility that regulation of gene expression by metabolic enzymes may be a novel mechanism by which Gram-positive bacteria, including *S. pyogenes*, coordinate multiple environmental cues, allowing essential transcription programs to be coupled with perceived nutritional status.

The EMBO Journal (2006) 25, 5414–5422. doi:10.1038/sj.emboj.7601393; Published online 26 October 2006

Subject Categories: signal transduction; microbiology & pathogens

Keywords: aldolase; LacD; regulation; SpeB; *Streptococcus*

Introduction

In eukaryotes, metabolic enzymes of the glycolytic pathway have been adapted to function as regulators of gene transcription. Examples include lactate dehydrogenase, glyceraldehyde-3 phosphate dehydrogenase, hexokinase and enolase, each of which can be translocated into the nucleus to act as components of transcriptional regulatory complexes. Typically, these enzymes function as metabolic sensors to couple cellular metabolism to gene regulation (for a review see Kim and Dang, 2005). How these enzymes have evolved into regulators is not understood. Given that many metabolic pathways are evolutionarily ancient, comparisons to prokaryotic organisms would be highly informative. However,

possible adaptation of prokaryotic metabolic enzymes for transcription regulation has not been well studied.

This paucity of information is surprising given the intimate relationship between the metabolisms of prokaryotes and their environment. Many pathogenic bacteria couple the sensing of specific nutrients with the regulation of genes that encode virulence proteins important for the interaction with host cells. Often, these nutritional cues are used to distinguish different temporal stages of the infection process. For example, the Gram-positive pathogen *Streptococcus pyogenes* (group A streptococcus), the causative agent of numerous human diseases ranging from pharyngitis and impetigo to the often fatal necrotizing fasciitis and septicemia (for a review see Cunningham, 2000), expresses numerous virulence genes in a highly ordered temporal pattern (Virtaneva *et al.*, 2005) that is influenced by nutrient availability (Chaussee *et al.*, 1997; Podbielski and Leonard, 1998; Smoot *et al.*, 2001). Temporal ordering is likely of critical importance to *S. pyogenes* pathogenesis, since numerous global transcription regulators that contribute to this process have been identified, and mutation of these typically results in large alterations of virulence (for a review see Kreikemeyer *et al.*, 2003). Thus, understanding how the regulatory network interrogates metabolic cues will be important for understanding the contribution of gene regulation to pathogenesis.

As a lactic acid bacterium, *S. pyogenes* relies exclusively on glycolysis for production of energy, suggesting that there are mechanisms to promote communication between metabolism and the virulence regulatory network. Many bacterial species monitor the activity of metabolic enzymes to control the hierarchical utilization of available sugars, a process known as carbon catabolite repression (CCR). In *Bacillus subtilis*, a sensory pathway that monitors the concentration of the glycolytic intermediate fructose-1,6-bisphosphate controls a transcription repressor known as CcpA. Greater than 80% of all genes regulated by glucose in *B. subtilis* are regulated by CcpA (Moreno *et al.*, 2001). Although CcpA homologs have been identified in many Gram-positive bacteria, there are important differences in the mechanisms of CCR. Furthermore, the role of CcpA may be more limited in the global pathways of carbon source regulation by Gram-positive pathogens. For example, in *Listeria monocytogenes*, a homolog of CcpA mediates catabolite control of some genes, but not carbon source regulation of virulence genes (Behari and Youngman, 1998). In contrast, an analysis of a streptococcal pathogen, *S. pneumoniae*, has shown that while CcpA contributes to virulence, it is not a global regulator of CCR (Iyer *et al.*, 2005). Thus, the pathways linking metabolism, CCR and virulence are not understood.

Our approach to understand how virulence and metabolism are connected in *S. pyogenes* has been to focus on a single virulence gene known to be highly expressed *in vivo*, and whose expression is sensitive to multiple nutritional cues during growth *in vitro*. In this regard, the gene encoding the secreted SpeB cysteine protease (*speB*) has been a useful

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Received: 12 May 2006; accepted: 26 September 2006; published online: 26 October 2006

model. While its specific role in virulence is unclear, it has been shown that *speB* is highly expressed in tissue during infection (Graham *et al.*, 2002; Cho and Caparon, 2005; Virtaneva *et al.*, 2005; Loughman and Caparon, 2006). In addition, transcription of *speB* is responsive to a number of cues during *in vitro* culture that are linked to nutrient availability, including bacterial cell density, carbon source depletion, pH, chloride and growth phase (Chaussee *et al.*, 1997; Podbielski and Leonard, 1998; Lyon *et al.*, 2001; Smoot *et al.*, 2001; Loughman and Caparon, 2006). Global profiling of the transcriptome in response to several of these cues revealed a large cohort of genes co-regulated with *speB* (Loughman and Caparon, 2006). Furthermore, the expression pattern of this group showed a significant correlation with that of *speB* *in vivo* (Loughman and Caparon, 2006). Thus, further analyses of the regulation of *speB* will likely reveal regulatory pathways with direct relevance to pathogenesis.

The observation that there are a large number of genes that are coordinately regulated with *speB*, in response to multiple signals suggested that there is a common pathway for sensing and responding to these cues. Therefore, a search was conducted for a gene that would regulate *speB* in response to carbon source availability, pH and chloride. This analysis resulted in the identification of LacD.1, a putative class I tagatose-1,6-bisphosphate aldolase similar to enzymes involved in galactose metabolism in Gram-positive bacteria. These data suggest that like eukaryotes, prokaryotic organisms have also adapted enzymes of central metabolism to roles in gene transcription. Further analyses of LacD.1 will provide insight into the pathogenesis of Gram-positive bacterial infections, and the molecular mechanisms used to adapt classical metabolic enzymes for management of a broad range of regulatory programs.

Results

LacD.1 is a negative regulator of SpeB activity

Known regulators of *speB* include RopB (also known as Rgg), a DNA-binding protein (Neely *et al.*, 2003) that is an essential activator of *speB* transcription (Lyon *et al.*, 1998; Chaussee *et al.*, 1999). However, RopB does not appear to directly sense environmental cues (Neely *et al.*, 2003; Loughman and Caparon, 2006). To search for additional regulatory factors, we screened for negative regulators of SpeB by examining colonies of a transposon mutant library on protease indicator media that had been buffered to pH 7.5. Since expression of *speB* requires a pH of less than 6.5 (Loughman and Caparon, 2006), any mutant expressing protease activity would arise from the inactivation of a negative regulator. Of approximately 1000 colonies analyzed, three expressed protease activity. These mutants were then tested on protease indicator plates containing 0.15 M NaCl. A single mutant with aberrant expression in response to both pH and NaCl was identified that contained a transposon insertion in an open reading frame (spy1704) that encodes a putative tagatose-1,6-bisphosphate aldolase annotated as LacD.1 (Figure 1A). In Gram-positive bacteria, LacD is part of the tagatose 6-phosphate pathway of lactose catabolism (van Rooijen *et al.*, 1991; Rosey and Stewart, 1992).

The importance of *lacD.1* was confirmed by the analysis of a LacD.1[−] mutant (JL151) containing an in-frame deletion allele. This mutant recapitulated the original mutant pheno-

type as follows: in contrast to the wild-type strain (Figure 1B; WT/none), the LacD.1[−] mutant produced protease under modified conditions of pH and salt (Figure 1B; LacD.1[−]/none). Activity was derepressed under the modified conditions to levels equivalent to WT under optimal conditions (Figure 1C; compare WT to LacD.1[−]). Introduction of a plasmid containing *lacD.1* complemented the mutant (Figure 1B; compare WT/vector to LacD.1[−]/pLacD.1) and restored sensitivity to the modified media (Figure 1C).

Consistent with a role as a negative regulator, overexpression of LacD.1 in the WT resulted in significant repression of SpeB under normally permissive conditions (Figure 1B; compare WT/vector to WT/pLacD.1) to levels about 65% less than WT alone (Figure 1C; $P < 0.001$). The more modest reduction of activity in the complemented mutant (approximately 30%)

Figure 1 LacD.1 is a negative regulator of SpeB activity. (A) Organization of the LacD.1 locus is shown. Arrows indicate the positions of the open reading frames. Gene names and genetic loci are indicated above and below the arrows, respectively, and are based on the genome of strain SF370 (Ferretti *et al.*, 2001). The position of a transposon insertion in *lacD.1* is indicated by the triangle. (B) A montage of colonies patched onto protease indicator media is shown. Protease activity is apparent as a zone of clearance surrounding bacterial growth. As indicated at the left, media was unmodified (Unmod.), buffered (pH 7.5) or supplemented with 0.15 M NaCl (NaCl). As noted above the figure, the behavior of HSC5 (WT) and mutant JL151 (LacD.1[−]) is shown in the absence of plasmid (none); pJL154, containing *lacD.1* (pLacD.1), or vector alone (vector). (C) Secreted protease activity for the indicated strains grown in various modified media is shown. Activity is reported as the percentage of WT activity in unmodified media. Data represent the mean and standard deviation of three independent experiments with samples analyzed in triplicate. An asterisk denotes a significant difference from WT/vector grown in the same medium ($P < 0.001$).

compared to WT (Figure 1C; LacD.1⁻/pLacD.1) is likely also due to overexpression of LacD.1. Taken together, these data establish that LacD.1 is a negative regulator of SpeB activity that participates in pathways responsive to both pH and NaCl.

LacD.1 mediates coordinate regulation by pH and NaCl

Analysis of transcription using real-time RT-PCR revealed that while the abundance of the *speB* transcript decreased in modified medium for WT, only a modest decrease was observed for the LacD.1⁻ mutant when compared to optimal medium (Table I), indicating that the mutant phenotype is due to derepression of *speB* transcription. A selection of other messages co-regulated with *speB* (Loughman and Caparon, 2006) was also probed. This panel of genes are either up- or downregulated greater than two-fold by pH and/or NaCl in WT, but were all less than two-fold different under these conditions in the mutant (Table I). In contrast, one gene (*ntpK*) whose expression is altered in response to NaCl, but not pH in WT, has a wild-type expression pattern in the mutant (Table I). These data demonstrate that there are at least two NaCl-sensitive pathways and implicate LacD.1 as a regulator of a subset of genes that are responsive to both pH and NaCl.

LacD.1 contributes to CCR

The involvement of LacD.1 in global regulatory pathways that coordinate virulence factor expression and carbohydrate metabolism (Loughman and Caparon, 2006) suggested that it might also mediate carbon source-specific regulation. To test the possible involvement of LacD.1 in CCR, the effect of glucose on *speB* transcription was tested. For WT, glucose repressed *speB* transcription greater than 50-fold (Figure 2A; *speB*, WT) similar to pH and NaCl (Figure 2B), but failed to repress transcription in the LacD.1⁻ mutant (Figure 2A), whose expression levels were similar to those observed with pH and NaCl (Figure 2B).

LacD.1 can function independently of CcpA

Our inspection of the known *speB* regulatory region (Neely *et al.*, 2003) revealed the presence of a candidate binding site for CcpA about 600 bases upstream of the *speB* start codon (data not shown). This raised the question as to whether LacD.1 pathway depends on CcpA. When compared, glucose also did not repress *speB* transcription in the CcpA⁻ mutant (Figure 2A), and did not repress protease activity on indicator

plates (Figure 2B; CcpA⁻/Glc). Expression of RopB was unaffected by the addition of glucose to the medium (Figure 2A; *ropB*) and there was also no obvious difference in growth between WT and the two mutants (data not shown). However, unlike the LacD.1⁻ mutant, the CcpA⁻ mutant retained its ability to respond to both pH and NaCl

Figure 2 Carbohydrates influence protease regulation through CcpA dependent and independent pathways. (A) Transcript abundance of *speB* and *ropB* for bacteria grown in medium supplemented with 0.15 M glucose relative to unmodified medium is shown, as determined by real-time RT-PCR. Compared are HSC5 (WT), JL151 (LacD.1⁻), and JL245 (CcpA⁻). Data represent the mean and standard deviation of three independent experiments analyzed in triplicate. (B) A montage of colonies patched onto protease indicator medium plates is shown. Strains are as described for (A), and media are as described in Figure 1. (C) Quantitative protease activity produced by the indicated strains following growth in the various carbohydrate-supplemented media is shown. Activity is reported as the percentage of activity for each strain in unmodified medium. Data represent the mean and standard deviation of three independent experiments, with samples analyzed in triplicate. An asterisk denotes a significant difference from WT grown in the same medium ($P < 0.001$). Abbreviations: Gro, glycerol; Glc, glucose; Lac, lactose; Gal, galactose; Fru, fructose; Mal, maltose; Man, mannose; and Scr, sucrose.

Table I LacD.1 mediates coordinate regulation by pH and NaCl in *S. pyogenes*

Locus ^a	Gene	Putative function	WT ^b		LacD.1 ^{-b}	
			pH	NaCl	pH	NaCl
Spy2039	<i>speB</i>	Cysteine protease	-664.5 ± 0.1	-567.7 ± 0.1	-9.7 ± 0.1	-4.1 ± 0.1
Spy1738	<i>manL</i>	Mannose PTS II	6.7 ± 1.8	3.7 ± 0.9	1.7 ± 0.3	1.8 ± 0.2
Spy0167	<i>slo</i>	Streptolysin O precursor	3.8 ± 1.0	5.1 ± 2.6	1.4 ± 0.2	1.1 ± 0.2
Spy0745	<i>sagH</i>	Streptolysin S biogenesis	2.50 ± 0.6	2.8 ± 0.3	1.5 ± 0.1	1.2 ± 0.2
Spy1915	<i>salA</i>	Lantibiotic precursor	-6.9 ± 0.1	-2.8 ± 0.3	-1.7 ± 0.1	-1.4 ± 0.1
Spy1815	<i>scrA</i>	Sucrose PTS II	-5.1 ± 0.1	-2.0 ± 0.3	-1.2 ± 0.1	1.3 ± 0.1
Spy0149	<i>ntpK</i>	Sodium ATPase	1.0 ± 0.5	6.4 ± 3.1	-1.5 ± 0.1	4.0 ± 0.1

^aGenomic loci, gene names, and putative function are based on current annotation of the genome of *S. pyogenes* SF370 (Genbank AE004092).

^bFold-change in transcript abundance for cultures grown to early stationary phase in buffered (pH) or salt supplemented-media (NaCl) versus cultures grown in unmodified medium as determined by real-time RT-PCR. HSC5 and JL151 are WT and LacD.1⁻, respectively. Data represent the mean and standard deviation of three independent experiments analyzed in triplicate.

(Figure 2B), suggesting that the pathways may function independently. To further explore this, protease expression was compared in response to a panel of carbohydrates. While the CcpA⁻ mutant retained the ability to repress *speB* in response to several sugars (e.g. galactose, fructose; Figure 2C), the LacD.1⁻ mutant was insensitive to all of the sugars tested (Figure 2C). These data suggest (i) that both CcpA and LacD.1 contribute to carbon source regulation; (ii) that LacD.1, but not CcpA, has a role in gene regulation in response to multiple signals; and (iii) that the LacD.1 pathway can function in the absence of CcpA, indicating that the regulators may influence gene expression through independent pathways.

Integration of LacD.1 and RopB circuits contributes to temporal regulation

Ectopic expression of *ropB* earlier in the growth cycle does not alter the temporal kinetics of *speB* expression (Neely *et al*, 2003). This suggests that a negative regulator, like LacD.1, restricts RopB activation of *speB* transcription until the appropriate signals are detected. This model predicts that constitutive expression of *ropB* in the absence of LacD.1 should permit protease production early in the growth cycle. To test this, a plasmid containing a constitutively expressed allele of *ropB* was introduced into the LacD.1⁻ mutant. This now allowed significant protease production at early time points (2–4 h), when activity is normally undetectable in WT (Figure 3; compare WT to LacD.1⁻ (pRopB), $P < 0.03$). This level of activity was about 50% of that observed at later time points, indicating that multiple factors are involved in regulation. Nevertheless, these data support a model for temporal regulation that requires activation by RopB in the context of derepression by LacD.1. Also, *lacD.1* itself is not regulated by growth phase (data not shown), suggesting that it must process a signal for derepression to occur.

To evaluate possible protein–protein interactions involving RopB and LacD.1, a co-immunoprecipitation strategy was employed using a strain (JL320) that overexpresses *lacD.1* due to the deletion of the gene encoding the LacR.1 repressor (Loughman and Caparon, submitted). This strain represses SpeB expression to an even greater extent than that obtained by ectopic overexpression of LacD.1 in WT, which should promote any RopB–LacD.1 interactions that contribute to repression. The chromosomal *lacD.1* locus in JL320 was further modified to express LacD.1 with a C-terminal FLAG tag, which was readily detectable in immunoblots of whole-cell lysates (Figure 3B, lane 3). Introduction of a plasmid expressing RopB-HA, followed by treatment with an α HA antiserum, resulted in the co-immunoprecipitation of LacD.1-FLAG (Figure 3B, lane 8). Co-immunoprecipitation of LacD.1-FLAG was specific since it was not observed in the absence of RopB-HA (Figure 3B; lanes 2, 4 and 6) and because RopB-HA did not co-immunoprecipitate a streptococcal protein that fortuitously cross-reacted with the α FLAG antibody (Figure 3B, lanes 6 and 8). In the reciprocal experiment, LacD.1-FLAG co-immunoprecipitated RopB-HA using an α FLAG antibody (data not shown). These data indicate that under conditions promoting repression of SpeB expression, RopB and LacD.1 can be found in an interacting complex.

Figure 3 Temporal regulation requires cooperation of RopB and LacD.1. (A) Secreted protease activity of the indicated strains as a percentage of WT activity in 8 h supernatant fluids is shown. Compared are HSC5 (WT), and JL151 (LacD.1⁻) in the presence or absence of a plasmid, pJL77, expressing *ropB* (pRopB). E64 is an inhibitor of SpeB cysteine protease activity. Data represent the mean and standard deviation for three independent experiments, with samples analyzed in triplicate. (B) RopB and LacD.1 form a complex. Shown is an immunoblot developed with an α FLAG antibody of untreated lysates (L) or lysates immunoprecipitated with an α HA antiserum (IP) from strains expressing various combinations of RopB-HA from a plasmid (pJL77) and LacD.1-FLAG from the chromosome, as indicated at the top of the figure. These strains included JL320 (LacR.1⁻), JL418 (LacR.1⁻, LacD.1-FLAG⁺), JL423 (LacR.1⁻, RopB-HA⁺) and JL425 (LacR.1⁻, LacD.1-FLAG⁺, RopB-HA⁺). The band corresponding to LacD.1-FLAG is indicated with a shaded arrow and a band corresponding to an unknown streptococcal protein that crossreacts with the α FLAG antibody, but does not co-immunoprecipitate with RopB, is indicated with an open arrow. Molecular mass of selected standards is shown on the left and is in kilodaltons.

LacD.1 is a unique regulatory aldolase

The tagatose 6-phosphate pathway has been best characterized in *Staphylococcus aureus*, *Streptococcus mutans* and *Lactococcus lactis*, where the enzymes are encoded in a seven gene operon, *lacABCDEFG* (Rosey *et al*, 1991; van Rooijen *et al*, 1991; Rosey and Stewart, 1992). However, the Lac.1 locus deviates considerably from this structure. It lacks *lacG*, encoding phospho- β -galactosidase, and the gene that should encode LacC.1 is truncated and contains several frameshift mutations. This structure is also conserved in all available *S. pyogenes* sequenced genomes. However, there is a second Lac locus in *S. pyogenes* (Lac.2) that closely resembles the canonical operon, encoding an aldolase, LacD.2, which is highly homologous (73% identical, 86% similar) to LacD.1 at the amino-acid level (Figure 4A). To evaluate whether LacD.1 was unique, a LacD.2⁻ mutant was analyzed and was found to possess a WT pattern of expression, repressing SpeB in response to pH and NaCl (Figure 4B; compare WT/none to LacD.2⁻/none) indistinguishable from

Figure 4 *LacD.1* is distinct from *LacD.2*. (A) Organization of the *LacD.2* locus. Arrows indicate the positions of the open reading frames. Gene names and genetic loci are indicated above and below the figure, respectively, and are based on strain SF370 (Ferretti *et al.*, 2001). (B) A montage of colonies patched onto protease indicator media is shown, as described for Figure 1. Strains analyzed include: HSC5 (WT), JL151 (*LacD.1*⁻), and JL251 (*LacD.2*⁻) in the absence of plasmid (none), pJL154 expressing *lacD.1* (pLacD.1), or pJL156, expressing *lacD.2* (pLacD.2). (C) Secreted protease activity in supernatant fluids derived from the indicated strains is shown. Media are as described for Figure 1 and the strains as described for (B). Activity is reported as the percentage versus the WT strain grown in unmodified medium. Data represent the mean of at least three independent experiments, with samples analyzed in triplicate. An asterisk denotes a significant difference from WT/none grown in the same medium ($P < 0.001$).

WT (Figure 4C). Furthermore, *LacD.2* could neither complement a *LacD.1*⁻ mutant (Figure 4B; compare *LacD.1*⁻/pLacD.2 to *LacD.1*⁻/pLacD.1) nor repress *SpeB* expression in WT when overexpressed (Figure 4B; compare WT/pLacD.2 to WT/pLacD.1). The *LacD* proteins were expressed to equivalent levels in both the WT and *LacD.1*⁻ strain as assessed by Western blotting of whole-cell extracts (data not shown). Thus, while *LacD.1* and *LacD.2* are closely related, only *LacD.1* can function in *SpeB* regulation.

Aldolase activity is not required for *LacD.1* regulation

Analysis of purified proteins revealed that both *LacD* proteins have similar aldolase activities against fructose-1,6-bisphosphate and were approximately 25% as active as rabbit muscle aldolase (Table II). Construction of enzymatically inactive *LacD.1* mutants took advantage of the high degree of conservation of active site structure between the tagatose- and

fructose-1,6-bisphosphate aldolases (Lorentzen *et al.*, 2004), including a catalytic lysine residue (K229) of rabbit muscle fructose-1,6-bisphosphate (St-Jean *et al.*, 2005) that forms the Schiff-base intermediate in the well-characterized reaction (Lai *et al.*, 1965). Alanine substitution of the corresponding residue in *LacD.1* (K204) resulted in a >98% decrease in aldolase activity (Table II; *LacD.1*_{K204A}). However, *LacD.1*_{K204A} retained an ability to complement the regulatory defect of the *LacD.1*⁻ mutant (Figure 5A; *LacD.1*⁻: compare *LacD.1* allele/K204A to *LacD.1* allele/WT) and repress *SpeB* activity in WT (Figure 5A; WT: compare *LacD.1* allele/K204A to *LacD.1* allele/WT). There were no significant differences in *SpeB* expression between *LacD.1* and *LacD.1*_{K204A} expressed in WT and in *LacD.1*⁻ backgrounds, respectively (Figure 5C). Thus, aldolase activity is dispensable for *LacD.1* regulatory function.

Substrate binding is implicated for regulatory function

The active site of class I aldolases is highly conserved across kingdoms (Lorentzen *et al.*, 2004). Further analysis of *LacD.1* using structural data of other class I aldolases revealed several conserved residues likely to interact with the C₄ hydroxyl and C₁ phosphate of the bisphosphate substrate (see Table II). Mutation of C₄ hydroxyl-interacting residues (Table II; K124A, E162A) produced *LacD.1* mutants that were completely defective in ability to complement a *LacD.1*⁻ mutant (Figure 5A; *LacD.1*⁻: compare *LacD.1* allele/K124A and E162A to vector), expressed protease activities identical to the mutant alone (Figure 5B), and could not repress activity when overexpressed in WT (Figure 5C). Based on a prior study (St-Jean *et al.*, 2005), mutation of C₁ phosphate-interacting residues utilized replacement with a bulky residue (Table II). One of these (R277A) was fully functional in regulation (Table II). However, the other two were either partially (G276F) or completely (A249F) defective for complementation (Figure 5A), protease activity (Figure 5B) or repression in WT (Figure 5C). All *LacD* proteins were expressed to equivalent levels in both the WT and *LacD.1*⁻ strain as assessed by Western blotting of whole-cell extracts (data not shown). These data establish that while *LacD.1* enzymatic activity is not required for regulatory ability, its ability to bind substrate likely plays a key role.

Discussion

Our examination of *speB* regulation revealed a role for a novel aldolase, *LacD.1*, in coordinating a transcriptional program in *S. pyogenes*. Thus, *LacD.1* becomes a prokaryotic member of an expanding list of metabolic enzymes that have been adapted to function in transcriptional regulation.

Of this list, *LacD.1* is the first aldolase that has been shown to have a function in regulation of transcription. However, there is a precedent for this role. For example, the binding of fructose-1,6-bisphosphate aldolase to actin has been implicated in several diverse processes involving cytoskeletal rearrangements (Wang *et al.*, 1996). In host-pathogen interactions, the fructose-1,6-bisphosphate aldolase of the apicomplexan parasite *Toxoplasma gondii* acts to bridge actin and cell surface proteins involved in gliding motility, a process that is essential for invasion into host cells (Jewett and Sibley, 2003). The dual role of these aldolases is often modulated by modifications such as phosphorylation (Zhong

Table II Summary of protein enzymatic and regulatory functions

Aldolase ^a	Mutation ^a	Aldolase activity ^b	Regulation competent ^c	Putative function ^d
LacD.2	None	2.370 ± 0.39	—	NA
LacD.1	None	2.638 ± 0.44	+	NA
	K204A (K229)	0.043 ± 0.03	+	Forms Schiff-base with C ₂
	K124A (K146)	0.095 ± 0.04	—	H-bonds with C ₄ OH [−]
	E162A (E187)	ND	—	H-bonds with C ₄ OH [−] , H ⁺ acceptor
	A249F (G272)	ND	—	H-bonds with C ₁ PO ₄ [−]
	G276F (G302)	ND	−/+	H-bonds with C ₁ PO ₄ [−]
	R277A (R303)	ND	+	H-bonds with C ₁ PO ₄ [−]

^aThe indicated aldolase contained no mutations (none) or was modified at specific positions as indicated. For LacD.1, the identities of the corresponding residues in AldoA (St-Jean *et al*, 2005) are indicated by parentheses.

^bActivity of purified protein was determined by assay of fructose-1,6-bisphosphate cleavage as described in Materials and methods. Data are presented in units/mg and represent the mean of at least three independent experiments analyzed in triplicate. Activity of AldoA in the assay was 11.59 ± 1.92 U/mg. ND, not determined.

^cCompetence (+) indicates that regulatory activity was not significantly different from WT; (−), that activity was not significantly different from vector alone; (−/+), that activity was significantly different from both WT and vector alone ($P < 0.05$), as assessed by pH and NaCl regulation upon expression in a LacD.1[−] mutant, and overexpression in WT.

^dFunction of the indicated residue in LacD.1 is predicted as noted based on biochemical and structural analyses of AldoA (St-Jean *et al*, 2005). NA, not applicable.

and Howard, 1990) or by substrate binding. In the latter case, binding substrate can induce large conformational changes in fructose-1,6-bisphosphate aldolase (St-Jean *et al*, 2005) that may be exploited to modulate protein–protein interactions to link regulatory activity to cellular metabolism.

Fructose and tagatose are nearly identical diastereomers that differ only in the rotation of the C₄ hydroxyl. Thus, it is not surprising that the fructose-1,6-bisphosphate and tagatose-1,6-bisphosphate aldolases have highly homologous active sites (Lai *et al*, 1965; Lebherz and Rutter, 1969) and produce identical products, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, which are key intermediates of glycolysis. This similarity suggests that LacD.1 may have been adapted to regulation similar to fructose-1,6-bisphosphate aldolase, by exploiting enzymatic activity, substrate recognition and/or protein–protein interactions. For the former, fructose-1,6-bisphosphate is the effector of the canonical pathway of CCR in Gram-positive bacteria, acting as an allosteric activator of a regulatory serine–threonine kinase (Reizer *et al*, 1998). The nearly identical tagatose-1,6-bisphosphate might also activate this pathway by exploiting the readily reversible aldolase reaction (Bissett and Anderson, 1980; Crow and Thomas, 1982) to link tagatose-1,6-bisphosphate concentration to nutritional status by the condensation of excess glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. However, several observations make this unlikely, including (i) that LacD.2 with an identical enzymatic activity could not complement a LacD.1[−] mutant; (ii) that LacD.1 enzymatic activity is not required for regulation; and (iii) that the LacD.1 and CcpA pathways do not overlap.

Since an enzymatic model is unlikely, it is possible that LacD.1 binds directly to DNA. However, it is also possible that LacD.1 interacts with a regulatory partner via protein–protein interactions that are modulated by binding substrate. This model is consistent with the observation that regulation requires residues important for substrate recognition and predicts that the distinction to LacD.2 resides in differences in surface-exposed residues involved in binding a partner. Supporting this hypothesis, our structural models place the majority of the non-homologous residues on the surfaces of the folded LacD.1 protein (Loughman and Caparon, unpublished data). A precedent for this model is regulation of

galactose metabolism in yeast, where a galactose kinase (GAL3) has been adapted to sense the intracellular concentration of galactose. Binding galactose may cause a conformational change that allows GAL3 to sequester a repressor (GAL80) that inhibits the DNA-binding activity of the GAL4 activator (reviewed in Bhat and Murthy, 2001). Similar to LacD.1, only the ability to bind substrate and not enzymatic activity is required for GAL3 regulatory function (Bhat and Murthy, 2001).

A GAL3-like model and the observation that LacD.1 acts as a repressor predict that a *speB* transcription activator is sequestered by LacD.1. The sensitivity of regulation to a range of carbohydrates suggests that LacD.1 senses its own products, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, the common intermediates of general carbohydrate metabolism. Also, since dihydroxyacetone phosphate is derived from the C₄–C₆ of tagatose-1,6-bisphosphate and mutations predicted to interact with the C₄ hydroxyl of tagatose-1,6-bisphosphate had a more pronounced effect on regulation, LacD.1 may be most sensitive to changes in dihydroxyacetone phosphate concentration. The interaction model predicts that under conditions of high carbon flux through glycolysis, the binding of its products induces a conformation in LacD.1 that allows it to sequester its regulatory partner, thus inhibiting the partner from activating its target genes.

An attractive candidate for this partner is RopB, which has also been implicated in growth phase-dependent regulation of metabolism and stress responses via repression of amino-acid metabolism (Chaussee *et al*, 2003). How RopB senses growth phase-related signals is unknown, but likely requires cooperation with a factor that senses changes in pH and nutritional status (Chaussee *et al*, 1997; Neely *et al*, 2003; Loughman and Caparon, 2006). The observation that the *speB* promoter is controlled by the presence of RopB only in the absence of LacD.1 and the ability to co-immunoprecipitate LacD.1 and RopB from streptococcal cells supports a RopB–LacD.1 interaction model. Whether this involves direct RopB–LacD.1 interaction or involves interactions with other proteins remains to be determined. However, RopB–LacD.1 cooperation would likely play a central role in the hierarchical utilization of carbohydrate or amino-acid

Figure 5 Mutation of catalytic and non-catalytic residues uncouples aldolase activity from regulatory activity. (A) A montage of colonies patched onto protease indicator media is shown, as described for Figure 1. Strains include HSC5 (WT) and JL151 (LacD.1⁻), containing pJL154 expressing *lacD.1* (WT), derivatives expressing the indicated mutant proteins (LacD.1 allele), or the vector alone (vector). Media are as described for Figure 1. (B) Secreted protease activity from JL151 (LacD.1⁻) expressing the various plasmids described in (A) is shown. Media are as described for Figure 1. (C) The LacD.1 overexpression phenotype in HSC5 (WT) is shown in the presence of vector or plasmids expressing unmodified (WT) or mutant LacD.1 proteins, as described for (A). For (B, C), activity is reported as the percentage of activity obtained for WT grown in unmodified medium. Data represent the mean of three independent experiments, with samples analyzed in triplicate. An asterisk denotes a significant difference from LacD.1⁻/vector (B) or WT/vector (C) grown in the same medium ($P < 0.05$).

substrates, consistent with the observation that both proteins cooperate to regulate expression of the SpeB protease, which may be important for scavenging peptides for metabolism.

The LacD.1 regulatory pathway processes other signals, including pH and NaCl. An acidic environment, produced by streptococcal fermentation or the host's inflammatory response, would have the effect of inhibiting the glycolytic pathway (Even *et al*, 2002; Raynaud *et al*, 2005) and thus, derepression of LacD.1-regulated genes. It is possible that pH is directly sensed by LacD.1, since the streptococci allow their cytoplasmic compartments to acidify at lower pH (Cotter and Hill, 2003), which may change the affinity of LacD.1 for its substrates. Changes in NaCl may have a similar effect, although how intracellular concentrations of NaCl are controlled by the streptococci is not well understood. Alternatively, there may be some other component of the regulatory circuit that acts upstream of LacD.1 and is responsible for sensing pH and NaCl.

In summary, this study has shown that a tagatose-1,6-bisphosphate aldolase has been adapted to regulate virulence gene expression in the bacterial pathogen *S. pyogenes*. Like adapted glycolytic enzymes in eukaryotes, it appears that streptococcal LacD.1 acts to couple transcription with nutrition by serving as a monitor of metabolic activity. Our preliminary examination of other Gram-positive genomes has shown that many also contain a second, incomplete Lac locus, suggesting that regulatory adaptation of metabolic enzymes may be widespread and underappreciated among the prokaryotes. Further analysis of LacD.1 will be useful for elucidating the mechanisms by which metabolic enzymes can be adapted for roles in transcriptional regulation.

Materials and methods

Bacterial strains and culture conditions

Molecular cloning experiments utilized *Escherichia coli* TOP10 (Invitrogen), and BL21(DE3) was used for expression of recombinant proteins. The *S. pyogenes* strain used was HSC5 (Hanski *et al*, 1992) and various mutant derivatives (Supplementary Table I) cultured at 37°C in C medium (Lyon *et al*, 1998) adjusted to pH 7.5 with NaOH. Various modified C media were utilized: buffered, by the addition of 1 M HEPES (pH 7.5) to a final concentration of 0.1 M; salt medium, containing 0.15 M NaCl, and carbohydrate-modified, containing various carbohydrates (see text) at final concentrations of 0.15 M. All components were added prior to autoclaving. Cultures on solid media were incubated under anaerobic conditions (Lyon *et al*, 1998). When appropriate, antibiotics were added at the following concentrations: kanamycin, 500 µg/ml and erythromycin 1 µg/ml for *S. pyogenes* and kanamycin, 50 µg/ml and erythromycin, 750 µg/ml for *E. coli*.

DNA and computational techniques

Transformation of *S. pyogenes* was performed as previously described (Caparon and Scott, 1991). Routine purification, manipulation and analyses of DNA were as described (Lyon *et al*, 1998). Amino-acid sequence alignments utilized the ClustalW method (Thompson *et al*, 1994). Statistical comparisons were made using the unpaired Student's *t*-test.

Mutagenesis strategy

Transposon mutagenesis utilized a modified version of Tn4001 (Lyon *et al*, 1998) containing an erythromycin resistance determinant (Miller and Neely, 2005). Construction of a library of transposon insertions was conducted as described (Lyon *et al*, 1998). For mutants of interest, the transposon insertion site was identified by direct sequencing of chromosomal DNA using a transposon-specific primer (IS256outR; Supplementary Table II) as described (Gibson and Caparon, 2002). Comparison to the genomic

database (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used to identify the site of transposon insertion. Additional sequence determination confirmed the identity of the locus in strain HSC5.

Protease activity assays

Solid media for the assay of proteolytic activity employed unmodified or modified C medium supplemented with 1.5% skimmed milk. Analysis of proteolytic activity in cell-free supernatants with the substrate fluorescein isothiocyanate-casein was conducted as described (Lyon *et al.*, 1998; Loughman and Caparon, 2006). The cysteine protease inhibitor E64 was added to selected samples to confirm that protease activity was due to SpeB (Lyon *et al.*, 1998). Values reported represent the mean and standard error of the mean for at least three independent experiments with samples analyzed in triplicate.

RNA isolation and real-time RT-PCR

Total RNA isolated from cultures at the onset of stationary phase (Loughman and Caparon, 2006) was analyzed by real-time RT-PCR as described (Brenot *et al.*, 2005) using the primers listed in Supplementary Table II. For the conditions used in this study, the range of abundance of *recA* transcript in samples with similar amounts of total RNA was less than two-fold; hence, transcript abundance was normalized to the abundance of *recA* as described previously (Brenot *et al.*, 2005). Data represent the means from a minimum of two experiments performed on different days in which RNA from at least three independent cultures was analyzed in triplicate.

Construction of deletion mutants and LacD.1-FLAG

All references to genomic loci are based on *S. pyogenes* SF370 genome (Ferretti *et al.*, 2001). The construction of mutants containing in-frame deletions in *lacD.1* (spy1704), *lacD.2* (spy1919), *ccpA* (spy0514), and *lacR.1* (spy1712) was performed by allelic replacement (Ji *et al.*, 1996) as previously described (Ruiz *et al.*, 1998) and utilized the primers listed in Supplementary Table II. Chromosomal structures of the resulting mutants were confirmed by sequence analysis of PCR products generated using the appropriate primers ('amplification primers'; Supplementary Table II). The mutant strains constructed were as follows: JL151 (HSC5 *lacD.1*_{Δ21-321}), JL251 (HSC5 *lacD.2*_{Δ18-323}), JL245 (HSC5 *ccpA*_{Δ2-331}), and JL320 (HSC5 *lacR.1*_{Δ2-248}). An epitope tagged version of LacD.1 was introduced into the *LacD.1* chromosomal locus of JL320 as described (Rosch and Caparon, 2005) using primers listed in Supplementary Table II. The resulting strain (JL418) was subsequently transformed with pJL77, which expresses an epitope-tagged version of RopB (RopB-HA), resulting in strain JL425. Transformation of JL320 with pJL77 created strain JL423.

Ectopic expression

DNA fragments containing *lacD.1* and *lacD.2* in the absence of their promoters were amplified using primer pairs JLP104 and JLP173 or JLP130 and JLP174, respectively (Supplementary Table II) and inserted under the control of the *rofA* promoter in pAGB5 as described (Meehl *et al.*, 2005). Expression of LacD.1 and LacD.2 in various *S. pyogenes* hosts (Supplementary Table I) was confirmed by Western blot analyses of whole-cell extracts (Rosch and Caparon, 2005) using a polyclonal αHA antiserum (Sigma) to recognize C-terminal epitope tags engineered with primers JPL173 and JPL174.

Immunoprecipitations

A 10 μl aliquot of polyclonal rabbit αHA serum (Sigma) was added to 50 μl of a 50% (v/v) protein A coated agarose bead slurry in PBS (Sigma). Following a 1-h incubation at 4°C, antibody-coated beads were washed twice with 1 ml binding buffer (200 mM NaCl in 50 mM sodium phosphate buffer (pH 7.5)). Streptococcal lysates were generated in binding buffer as described (Rosch and Caparon, 2005), 200 μl added to the washed beads, and incubated at 4°C for 15 h. Beads were washed five times with 1 ml binding buffer, and

the protein-coated beads were dissolved in 50 μl of protein loading buffer. Proteins were resolved by SDS-PAGE and transferred to PVDF membranes for Western blotting with mouse monoclonal αFLAG antibody (1:5000 final dilution) and peroxidase conjugated goat αmouse secondary antibody (1:10 000 final dilution; both from Sigma).

Construction of mutant LacD.1 enzymes

The single amino-acid substitution mutations indicated in the text were introduced into LacD.1 with the QuikChange site-directed mutagenesis kit (Stratagene), using the primers described in Supplementary Table II and pJL154 as template. DNA sequence analyses using primers flanking *lacD.1* (Supplementary Table II) were conducted to confirm the expected sequences. Following transformation of wild type or LacD.1[−] (JL151) hosts with the resulting plasmids (Supplementary Table I), expression and stability of the mutant proteins was determined by Western blot analysis as described above.

Construction of LacD expression plasmids

The *lacD.1* or *lacD.2* open reading frame was amplified by PCR from genomic DNA with primers JLP165 and JLP166, or JLP167 and JLP168, respectively (Supplementary Table II). The resulting fragments were digested with *NcoI* and *XhoI* and inserted between the *NcoI* and *XhoI* sites of pET-24d (Novagen), which produced an in-frame fusion with codons for the C-terminal six-Histidine tag encoded by the vector. DNA sequencing confirmed the fidelity of these constructs, which were designated pJL132 (LacD.1) and pJL134 (LacD.2). The K124A and K204A mutations were introduced into *lacD.1* on pJL132 as described above.

Purification of enzymes

E. coli containing expression plasmids were grown to mid-exponential phase (OD₆₀₀ = 0.8) in M9 minimal medium containing 0.4% (w/v) glucose and 50 μg/ml kanamycin at 37°C and induced with IPTG (1 mM). Following incubation for 3 h at 30°C, cells were harvested and the six-His-tagged proteins purified by chromatography over a nickel affinity resin, according to the recommendations of the manufacturer (Qiagen). The eluted protein was dialyzed against storage buffer (50 mM HEPES (pH 7.5), 1 mM EDTA) to remove the imidazole. The purity of the preparations, as assessed by SDS-PAGE and staining with Coomassie brilliant blue, was greater than 95%. Protein concentrations were determined by the bicinchoninic acid method using commercial reagents (Sigma) with bovine serum albumin as a standard.

Aldolase activity assays

The enzymatic activity of purified enzymes was determined using a fructose-1,6-bisphosphate substrate (Sigma, catalog #47810) in a direct assay based on Boyer's modification of the hydroxine assay (Jagannathan *et al.*, 1956). Activity is presented in units/mg and is calculated using the following equation: units/mg = (ΔA₂₄₀/minute)/(mg enzyme/ml reaction mixture). For reference, rabbit muscle fructose-1,6-bisphosphate aldolase (Sigma, catalog #A-2714) was also analyzed in the assay.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Craig Smith for providing assistance with protein alignment and structure prediction. We also thank Jonathan Lenz for technical assistance. This work was supported by Public Health Service grants AI046433 and AI064721 from the National Institutes of Health.

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