Identification and Characterization of the *pckA* Gene from *Staphylococcus aureus*[†]

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The *Staphylococcus aureus pckA* gene was identified and characterized. A *pckA* mutant lacked detectable phosphoenolpyruvate carboxykinase activity and grew poorly in the absence of glucose. Both enzymatic activity and *pckA* promoter activity in wild-type cells grown in the absence of glucose were at least 22-fold greater than activities in cells grown in the presence of glucose.

In a search for histidine kinase genes from Staphylococcus aureus, a 227-nucleotide PCR-generated DNA fragment (originally designated kin50) that had strong sequence similarity to the phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) gene from other organisms was identified (1). Since PEPCK is an essential enzyme for gluconeogenesis, it likely plays a key role in the growth and survival of S. aureus cells in the absence of glucose. Therefore, to investigate whether this PCR fragment originated from within the staphylococcal pckA homolog, it was used to mutate S. aureus RN4220 (6) by Campbell integration (2), creating strain KB501. As shown in Fig. 1, RN4220 ($pckA^+$) exhibited similar growth in the presence and absence of glucose (maximum turbidities of 295 and 269 Klett units, respectively). On the other hand, the growth yield of KB501 was dramatically reduced in the absence of glucose compared with that in its presence (maximum turbidities of 128 and 250 Klett units, respectively). This glucose-specific defect observed for KB501 suggests that gluconeogenesis is affected in this strain and is consistent with the hypothesis that pckA encodes PEPCK.

PEPCK assays. To determine PEPCK activity, strains RN4220 and KB501 were grown in the presence and absence of glucose and PEPCK assays were performed. Cell extracts of S. aureus were prepared by suspending exponentially growing cells (from 50-ml cultures) in 1 ml of 50 mM imidazole (pH 7.0)-5 mM MnCl₂-1 mM β-mercaptoethanol. Lysostaphin and DNase I were added to final concentrations of 50 and 10 μ g/ml, respectively, and samples were placed at 37°C for 10 min. Samples were then clarified by passage through a 20gauge needle and centrifuged at $15,000 \times g$ for 15 min at 4°C. Supernatant fractions were assayed for PEPCK activity spectrophotometrically by monitoring oxaloacetate production via a coupled system as described previously (9). The reaction mixture contained 100 mM imidazole (pH 7.0), 50 mM NaHCO₃, 10 mM PEP, 5 mM Na₂ADP, 1 mM MnCl₂, 5 mM dithiothreitol, 300 µM NADH, 8 U of malate dehydrogenase (Sigma), and 0.1 ml of crude extract (0.5 to 2 mg of protein) in a final volume of 1.0 ml. The initial velocity of NADH oxidation (without PEP) at 37°C was monitored for 5 min at 340 nm after the reaction was initiated with extract. PEP was then added, and NADH oxidation was again monitored for 5 min.

* Corresponding author. Mailing address: Center of Marine Biotechnology, Columbus Center, Suite 236, 701 E. Pratt St., Baltimore, MD 21202. Phone: (410) 234-8875. Fax: (410) 234-8899. Electronic mail address: bayles@mbimail.umd.edu. When cells were grown in the absence of glucose, PEPCK activity was detected in RN4220 (22.0 \pm 3.3 nmol/min/mg of protein; mean \pm standard deviation) but was undetectable in KB501 (<0.3 nmol/min/mg of protein). The absence of detectable PEPCK activity in KB501 is consistent with the assertion that plasmid integration disrupted the gene encoding this enzyme. In the presence of glucose, PEPCK activity was undetectable (<0.3 nmol/min/mg of protein) in both RN4220 and KB501, indicating that PEPCK activity is glucose repressible. The inability to detect PEPCK in RN4220 cultures grown in the presence of glucose is similar to results obtained for *Bacillus subtilis* (4).

Cloning and sequence analysis. DNA flanking the integrated plasmid in KB501 was cloned using a plasmid rescue



FIG. 1. Growth of strains KB501 (*pckA*) and RN4220 in the presence and absence of glucose. Results are representative of four independent experiments. Culture turbidity was measured with a Klett-Summerson colorimeter (filter no. 66); 1 Klett unit corresponds to $\sim 10^6$ CFU/ml.

[†] Contribution 265 from the Center of Marine Biotechnology.

TTTANATTTATATTACGANAATTCTCTCTCTGTGAGCTANATANAAAGACCTTCTAACTATTAATATAGAGAGAAGGCCTAATACGTCC	90
attegetettategeteagacctattegetegeaaccettettettataaaaaagaggtegetgegetteattgggteeaaccettettettataaaaaagaggtegetgggtegetgetgggtegetgetgegeteget	180
TGTCCCTCCACCACTCAGGATAAGAGAATCCGTTAAAAATAATAGTACCTAATTAAT	270
AGTAAAATATTGTAGATTAATTATGTTAATGTGTTATACTAATTAAA <u>TGTAAAGGCTTACA</u> TTTAAATTATCGCTTT <u>GGAGG</u> GATTTAGG	360
S.D. ATGTCAGTAGACACATACACTGAAACAACTAAAATTGACAAATTACTGAAAAAACCAACGTCACATTTTCAACTTGACGACACAACTT M S V D T Y T E T T K I D K L L K K P T S H F Q L S T T Q L	450 30
TATAATAAAAATCTTAGACAATAACGAAGGGGTATTAACGAGAACTTGGGGCGGTGTGATGACAGGACAGGAAAATATACGGGCGTTCGCCTYN N K I L D N N E G V L T E L G A V N A S T G K Y T G R S P	540 60
AAAGACAAATTTTTTTGTCTCTGAACCTTCATATAGAGATAACATTGATGGGGGGAAATTAATCAACCTATCGATGAAGAAACTTTCTTGTG K D K F F V S E P S Y R D N I D W G E I N Q P I D E E T F L	630 90
AAGTTATACCATAAAAGTACTAGACTATTTAGATAAAAAAGATGAACTATACGTATTTAAAAGGCTACGCTGGTAGCGATAAAGATACAATG	720
Κ L Υ Η Κ Υ L D Υ L D Κ Κ D Ε L Υ Υ F Κ G Υ A G S D Κ D Τ Μ	120
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ACTGTAGTTGCAGAAGATGGTTCAGTGGACTTTGAAGACAATCGTTATACAGAAAACACGCGTGCCGCTTATCCAATTAATCACATTGAC T V V A E D G S V D F E D N R Y T E N T R A A Y P I N H I D	1350 330
ANTATTGTAGTACCATCTAAAGCAGCACATCCAAATACAATTATTTTCTTAACTGCGGATGCATTTGGTGTTATTCCACCGATTTCAAAG	1440
N I V V P S K A A H P N T I I F L T A D A F G V I P P I S K	360
TTAAATAAAGACCAAGCAATGTATCATTTCTTGAGTGGTTTCACTTCTAAATTAGCTGGTACAGAGCGTGGTGGAGCAGAACCTGAACCALNKDQAACCTGAACCALNKDQAACCTGAACCALNKDQAACCTGAACCALNKDQAACCTGAACCALNKDQAACCTGAACCA	1530 390
TCATTCTCAACATGTTTCGGAGCACCGTTCTTCCCGTTACACCCTACTGTTTACGCTGATCTATTAGGTGAACTTATCGATTTACGATTACATGAT S F S T C F G A P F F P L H P T V Y A D L L G E L I D L H D	1620 420
GTTGATGTTTATCTTGTTAATATGGACGGAGAGAATATGGTGTAGACGTAGAATCAGCTAAATACGTAAATGGTGTAGACGTAGAATCAGCTAAATGGTGTAGACGTAGAATCAGCTAAATGGTGTAGACGTAGAATCAGCTAAATGGTGTAGACGTAGAATCAGCTAAATGGTGTAGACGTAGAATCAGCTAAATGGTGTAGACGTAGAATCAGCTAAATGGTGTAGACGTAGAATCAGCTAAATGGTGTAGACGTAGAATCAGCTAACAATGGTGTAGACGTAGAATCAGCTAACAATGGTGTAGACGTAGAATCAGCTAACAATGGTGTAGACGTAGAATCAGCTAACAATGGTGTAGACGTAGAATCAGCTAACAATGGTGTAGACGTAGAATCAGCTAACAATGGTGTAGACGTAGAATCAGCTAACAATGGTGTAGACGTAGAATCAGCTAACAATGGTGTAGAATCAGCGTAGAATCAGCTAACAATGGTGTAGACGTAGAATCAGCTAACAATGGTGTAGACGTAGAATCAGCTAACAATGGTGTAGACGTAGAATCAGCGTAACAATGGTGTAGAATGGTGTAGGACGTAGAATCAGCTAACAATGGTGTAGGACGTAGAATCAGCTAACAATGGTGTAGGACGTAGAATCAGCTAACAATGGTGTAGGACGTAGAATCAGCTAACAATGGTGTAGGACGTAGAATCAGCTAACAATGGTGTAGGACGTAGAATCAGCGTAGAATCAGCGTAACAATGGTGTAGGACGTAGAATGGTGTAGGACGTAGAATGGTGTAGGACGTAGAATGGTGTAGGACGTAGAATGGTGTAGGACGTAGAATGGTGTAGGACGTGAGGAGGTAGAATGGTGTAGGACGTAGAATGGTGTAGGACGTGAAGAATGGTGTAGGACGTAGAATGGTGTAGGACGTAGAATGGTGTAGGACGTGAAATGGTGGAGGTAGAATGGTGGAGGTGAAGAATGGTGAATGGTGAGGAG	1710 450
- AACCAAGCGATTTCTGGCAAATTGAAAAATGCAGAATATACAAAAGATAGTACGTTTGGTTTAAGCATTCCTGTAGAAAATTGAAGATGTA	
	1800
N Q A I S G K L K N A E Y T K D S T F G L S I P V E I E D V	1800 480
N Q A I S G K L K N A E Y T K D S T F G L S I P V E I E D V CCGAMAACAATTTTAAATCCAATTAAAGCTCGGGGGGGAGAAATATAAAGCACAAGGAGAATTTTAATCCAACGTTTGAAAAG P K T I L N P I N A W S D K E K Y K A Q A E D L I Q R F E K	1800 480 1890 510
N Q A I S G K L K N A E Y T K D S T F G L S I P V E I E D V CCGAMACAATTTTAAATCCAATTAAAGCTTGGACGGACAAAGAGAAATATAAAGCACAGGAGAATTTAAATCCAACGTTTTGAAAAG P K T I L N P I N A W S D K E K Y K A Q A E D L I Q R F E K AACTTCGAAAAATTTGGTGAACATATTGGTGAACATATGGTAGGTA	1800 480 1890 510 1980 530

FIG. 2. Nucleotide sequence and deduced amino acid sequence of the *pckA* gene from *S. aureus*. The nucleotide sequence of the *pckA* gene was determined by the dideoxy chain termination method of Sanger et al. (10) and the Sequences DNA sequencing kit (United States Biochemical, Inc.). Sequence-specific oligonucleotide primers were synthesized with an Expedite 8909 nucleotide synthesizer and were utilized to sequence both strands of pKIN50P and pKIN50E. A computer analysis of sequence data was conducted with the GCG Wisconsin Computer Package Unix-Version 8.0 (3). A putative Shine-Dalgarno sequence (S.D.), a potential CcpA binding site (underlined), and inverted repeats (arrows) are indicated. The asterisk indicates the apparent transcription start site. The PCR product that was amplified (designated *kin50* [1]) and used as a site of homology for Campbell integration is shaded.

technique (2) resulting in upstream (pKIN50P) and downstream (pKIN50E) clones containing 2.3- and 5.9-kb inserts, respectively. Sequence analysis of the cloned region revealed an open reading frame with the potential to encode a 530amino-acid protein (Fig. 2). Comparison of the deduced amino acid sequence of this protein with the amino acid sequences in the GenBank database demonstrated extensive sequence identity to PEPCK from *Rhizobium meliloti* (48.8%; accession no. U15199), *Saccharomyces cerevisiae* (48.6%; accession no. X13096), *Escherichia coli* (47.2%; accession no. M59823), and *Trypanosoma cruzi* (43.4%; accession no. M91163). This analysis, along with the PEPCK assays described above, strongly suggests that *pckA* encodes PEPCK.

Transcription analysis. To examine expression of pckA, a DNA fragment containing the pckA promoter region (nucleotides 1 to 408) was amplified by PCR and cloned in plasmid pLC4 (8), generating a transcriptional fusion to the *xylE* gene. This plasmid, designated pWS50, was then introduced by transformation into strain RN4220. As an initial test of promoter activity, RN4220(pWS50) was grown on nutrient agar

plates with and without glucose. Catechol 2,3-dioxygenase activity was detected by spraying colonies with catechol after 18 h of growth. Colonies formed in the presence of glucose remained white, while those formed in the absence of glucose turned intensely yellow, an indication of catechol 2,3-dioxygenase activity (data not shown). On the other hand, RN4220 (pLC4) (XylE⁻) formed colonies that were white in both the presence and absence of glucose.

To obtain a quantitative measure of promoter activity, spectrophotometric catechol 2,3-dioxygenase assays were performed as described by Sheehan et al. (11). Fresh overnight cultures were used to inoculate 15 ml of nutrient broth containing chloramphenicol (5 μ g/ml) with or without 0.5% glucose and grown to late exponential phase. Catechol 2,3-dioxygenase activity in RN4220(pWS50) grown in the absence of glucose was 8.7 mU/mg, compared with 0.4 mU/mg (one milliunit corresponds to the formation of 1 nmol of 2-hydroxymuconic semialdehyde per min at 30°C [13]), which was obtained from cultures grown in the presence of glucose, a 22.4-fold reduction. Strain RN4220(pLC4) showed similar low levels of



FIG. 3. Primer extension analysis. An oligonucleotide primer complementary to the 5' end of the pckA coding region (nucleotides 463 to 496) was used as a primer for reverse transcription as described previously (2). The size of the extended product (81 bases) was determined by comparison with a DNA sequencing ladder of the pckA promoter region by using the same primer.

activity in the presence and absence of glucose. The regulation by glucose was similar to that observed for PEPCK activity in RN4220 and demonstrated that *pckA* expression is under catabolite repression control, similar to PEPCK-encoding genes from other bacteria (5, 7).

Analysis of the DNA sequence upstream from pckA (Fig. 2) revealed the presence of an inverted repeat sequence that was nearly identical (9 of 10 nucleotides) to the consensus CcpA binding site (a control sequence for catabolite repression) from *B. subtilis* (12). The presence of such a site in the promoter region of pckA (Fig. 2) suggests the existence of a CcpA-like protein responsible for glucose repression in *S. aureus*. A primer extension analysis (Fig. 3) demonstrated that the start site for transcription originated at an adenine residue located 40 bp upstream from the putative translation start site (Fig. 2). The relationship between the transcription start site and CcpA consensus sequence suggests that catabolite repression of pckAis mediated by the binding of a CcpA-like regulatory protein at this site. Nucleotide sequence accession number. The sequence of pckA has been deposited in GenBank under accession no. L42943.

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