

Unclassified

ARO 12368.2-4

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER DAAG29-76-G-0288	2. JOINT ABSESSION NO. 3. RESEARCH CATALOG NUMBER (Final rept. 1 Jul 74-30 Jun 77)	
4. TITLE (and Subtitle) THE EFFECT OF CYCLIC AMP ON THE PERMEABILITY CHARACTERISTICS OF THE <u>ESCHERICHIA COLI</u> MEMBRANE SYSTEM	5. TYPE OF REPORT & PERIOD COVERED Final Report (07-01-74-1-06-30-77)	
7. AUTHOR(s) (10) Walter J. Dobrogosz	6. PERFORMING ORG. REPORT NUMBER DAAG29-76-G-0288	
9. PERFORMING ORGANIZATION NAME AND ADDRESS North Carolina State University Department of Microbiology Raleigh, North Carolina 27607	8. CONTRACT OR GRANT NUMBER(s) U.S. DAHC04-74-G-0242 (15) DAHC04-75-G-0196 DAAG29-76-G-0288	
11. CONTROLLING OFFICE NAME AND ADDRESS U. S. Army Research Office Post Office Box 12211 Research Triangle Park, NC 27709	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 11. REPORT DATE (11) 21 September 1977	
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) Same (18) ARO (19) 12368.2-4	13. NUMBER OF PAGES 17 (1239)	
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited.	15. SECURITY CLASS. (of this report) Unclassified	
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE	
18. SUPPLEMENTARY NOTES The findings in this report are not to be construed as an official Department of the Army position, unless so designated by other authorized documents.		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Regulation of membrane synthesis cyclic AMP regulation cyclic AMP regulation of membrane synthesis cyclic AMP and transport activities <u>E. coli</u> membranes and cyclic AMP		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Various metabolic functions associated with the <u>Escherichia coli</u> membrane system were analyzed in mutant strains lacking adenylate cyclase (<u>cya</u> mutations) or cyclic AMP binding protein (CRP) activity (<u>crp</u> mutations). Mutations which have suppressed the organism's requirement for cyclic AMP were also studied, and mutants of <u>Salmonella typhimurium</u> were constructed which are <u>cya</u> or <u>crp</u> in addition to lacking the enzymes of the phosphoenolpyruvate: phosphotransferase system (PTS). These studies have established that key		

(2)

B.S.

DDC

RECEIVED
OCT 28 1977
B

ADA 045952

DDC FILE/COPY

410448

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

19. KEY WORDS:

bioenergetic regulation in E. coli
transport and energetic functions in E. coli
Escherichia coli and cyclic AMP regulation

20. ABSTRACT:

cont.

transport and bioenergetic activities of the enterobacterial membranes are regulated at the genetic level of transcription by the cAMP-CRP regulatory complex. The ability of E. coli to transport substrates via PTS and non-PTS routes are under this control. The organism's ability to generate a proton motive force (PMF) and to link this force to transport activities and ATP synthesis are also under this control. All parts of the E. coli envelope, the inner, cytoplasmic membrane, the periplasmic region, and the outer membrane are to one degree or another affected by the cAMP-CRP regulatory function.

ACCESSION	by
NTIS	Section IV
DDC	Section IV
UNANNOUNCED	
REPLICATION	
BY	
DISTRIBUTION AVAILABILITY CODES	
Dist. STATE Int. or SPECIAL	
A	

Best Available Copy

TITLE

The Effect of Cyclic AMP on the Permeability Characteristics
of the Escherichia coli Membrane System

TYPE OF REPORT (TECHNICAL, FINAL, ETC.)

Final Report

AUTHOR (S)

Walter J. Dobrogosz
Professor of Microbiology

DATE

September 21, 1977

U. S. ARMY RESEARCH OFFICE

CONTRACT / GRANT NUMBER

DAHCO4-74-G-0242 7/1/74-6/30/75
DAHCO4-75-G-0196 7/1/75-6/30/76
DAAG29-76-G-0288 7/1/76-6/30/77

INSTITUTION

North Carolina State University

APPROVED FOR PUBLIC RELEASE;
DISTRIBUTION UNLIMITED.

Table of Contents

I. Introduction	1
II. Summary of Research	
1. General procedures	2
2. Transport of glucose-6-phosphate	3
3. Role of cAMP-CRP in the PTS.	4
4. Construction of PTS and cAMP-CRP "double" mutants. . .	4
5. Studies on membrane energetics	5
6. Role of cAMP-CRP in construction and function of the <u>E. coli</u> outer membrane and periplasmic proteins. .	7
7. Fatty acid content under cAMP-CRP control.	8
8. Genetic suppression of the cAMP requirement.	9
9. Transport studies using the <u>psm</u> mutation	10
10. Progress resume.	10
III. Publication List and Bibliography to Summary	12
IV. Personnel Associated with Project.	15

I. Introduction

This final report is prepared according to the guidelines described in RDRD-CS Form 18 (2 July 1973). A number of reprints of published work have already been submitted and all others will be forwarded as soon as they become available. A listing of all publications and abstracts derived from work supported by these ARO contracts is presented in Section III of this report. Since all technical data will be presented as published works, the substance of this final report is presented as the overall summary described in Section II.

II. Summary of Research - Covering Period of July 1, 1974 - June 30, 1977.

1. General procedures.

The transcription of certain inducible enzyme systems in Escherichia coli does not occur unless the promoter sites for these operons are activated by a protein - the catabolite repression protein (CRP) - which must be allosterically modified to its active, binding conformation by a low molecular weight cyclic nucleotide identified as cyclic AMP (cAMP). This regulatory phenomenon is known as catabolite repression (CR) and involves synthesis of transport proteins and dissimilatory enzymes that are utilized in the catabolism of substrates such as lactose, glycerol, the pentoses, and many others. The specific inducers for each of these operons (e.g., 3-galactosides for lactose dissimilation, etc.) and the cAMP-CRP complex must be simultaneously available if transcription is to be initiated. When cells susceptible to CR are grown on other substrates such as glucose, gluconate, or glucose-6-phosphate their production of cAMP is inhibited resulting in lowered cAMP-CRP levels and a consequent repression of those transcriptions requiring activation by this complex. In other words, the amount of cAMP produced under these growth conditions is insufficient to produce the cAMP-CRP conformation needed to allow certain operons to be transcribed. Other catabolite repressible operons, however, apparently continue to be transcribed until the intracellular cAMP pool is lowered even further as occurs in gln mutant strains in which adenylate cyclase is not synthesized. Mutants unable to synthesize the CRP protein (crp mutants) are also unable to synthesize catabolite repressible proteins.

In wild type cells CR can be overcome by adding exogenous cAMP to the culture medium containing the repressor substrates. In cya mutants, exogenous cAMP must be added to the culture medium in order for the cells to synthesize the catabolite repressible proteins, and in crp mutants these proteins are not synthesized regardless of availability of cAMP. In studying systems suspected of requiring the cAMP-CRP complex it is necessary to use these two mutant strains in addition to the wild type, parental cells. This was done in the research summarized here. The objective of these studies was to determine the extent to which the membrane functions of E. coli, particularly those involving transport activities, were regulated by the cAMP-CRP regulatory system. Although these studies are far from complete excellent progress was made and a cohesive body of new information was generated during the period covered by this contract. If all manuscripts yet outstanding are completed and accepted for publication within the next few months, a total of 11 papers and 4 abstracts of papers presented at the national meetings of the American Society for Microbiology will have been generated as a result of this contract.

2. Transport of glucose-6-phosphate. Transport proteins involved in metabolism of substrates such as glycerol, lactose, and the pentoses are synthesized under cAMP-CRP regulation. We have established that the hexose phosphate transport (HPT) system used for glucose-6-phosphate (G6P) uptake is also regulated in this fashion (2,9). We demonstrated that there was only one HPT system in E. coli cells - a point of confusion based on earlier literature, and that

prior confusion concerning the apparent K_m for G6P transport could be attributed to the fact that different researchers used different concentrations of phosphate in their uptake assay systems. We showed that the K_m for G6P uptake increased linearly with the increase of phosphate. Also, we demonstrated that it was critical to purify commercial radioactive G6P before use in transport studies. Other workers have apparently not done so resulting in some misleading information in the literature.

3. Role of cAMP-CRP in the PTS. While it was not surprising that synthesis of the HPT required cAMP-CRP, we were surprised to discover that the phosphoenolpyruvate: phosphotransferase system (PTS) was also under this control. Cya and crp mutants were found to be unable to synthesize the high affinity (K_m ca 10 μ M) transport system for glucose (2,4,5). Cya cells synthesized this latter system only if cAMP was added to the growth medium; crp mutants were unable to synthesize this system under any circumstance. This finding raised questions concerning the nature of the regulatory interactions that exist between the PTS and the cAMP-CRP systems in these bacteria. Others had already shown that the PTS inhibited cAMP synthesis by controlling adenylate cyclase activity. Our data suggest this regulation is cyclic in nature with cAMP-CRP required for the cells to synthesize factors 111 and 11 b' which are believed to be the components unique to the high affinity PTS.

4. Construction of PTS and cAMP-CRP double mutants. We have proposed that if the cyclic nature of this interaction is to be understood at the mechanistic level it will be necessary to analyze mutant

strains that lack components of the PTS and the cAMP-CRP systems. In this connection we undertook construction of mutants lacking both systems. For this purpose, Salmonella typhimurium strains having well characterized single and extended deletions of the PTS were used along with strains of this organism mutated in the cya or crp genes. Through appropriate transduction experiments we were able to construct strains having the following genotypes: pts I V cya, pts I V crp, pts H cya, pts H crp, pts I err V cya, pts HI V cya, pts HI V crp, pts HI err V cya. These "double" mutants have now been well characterized and studied with respect to their ability to synthesize the anaerobic, inducible biodegradative threonine deaminase (14). These studies are still incomplete and must include assays for cAMP production and L-threonine transport capacities in all strains. To date, however, our data with these "double mutants" indicate that the pts II and pts I gene products somehow represent the organism's ability to synthesize the biodegradative threonine deaminase which is regulated by the cAMP-CRP complex. We anticipate that valuable information will be obtained when these new "double" mutants are examined in further detail.

5. Studies on membrane energetics. Transport of substrates across the bacterial membrane must be studied in terms of the energetics involved in the process. In the case of PTS substrates the mechanism of transport is vectorial phosphorylation in which phosphoenolpyruvate (PEP) is the energy donor. Transport of other substrates such as methionine or glutamine utilize ATP directly in some manner. The majority of substrates transported under aerobic conditions enter the cells utilizing a proton motive force (PMF) consisting of H^+ and ionic

gradients established across the membrane during exergonic dissimilations. Specific symport proteins (translocators) transport the substrates into the cells as energized by the PMF. The formation of this PMF is also believed responsible for synthesis of intracellular ATP via the oxidative phosphorylation coupling of electron transfer to the ATPase reaction: $\text{P}_i + \text{ADP} \rightarrow \text{ATP}$.

Our studies have shown that cAMP-CRP regulation plays an important role in these PMF-associated membrane functions in E. coli. We have established that this complex is needed for 1) normal synthesis of the organism's cytochrome system (1,5), 2) formation of the primary dehydrogenases: NADH dehydrogenase, D-lactate dehydrogenase, and succinate dehydrogenase, 3) reduction of O_2 by the substrates: NADH, D-lactate, and succinate, 4) formation of the O_2 -linked transhydrogenase and 5) synthesis of the flavin cofactors: FMN and FAD (7). FAD levels in cya and crp mutant cells, for example, were only 10% of wild-type levels (7). The naphthoquinone levels in these cells seem unaffected by these mutations. From these data we concluded that the major components of the E. coli electron transport system are regulated by cAMP-CRP.

Continuing along these lines, we were then also able to show that this regulatory complex was very much involved in the ability of E. coli to generate a PMF from D-lactate and to use this PMF for ATP synthesis. ATPase concentrations per se seemed unaffected by this complex. When the artificial energy source - phenazine methosulfate-aerobate - was used to energize the PMF instead of D-lactate, the cya and crp mutant cells were found to be totally

unable to form a wild-type PMF or generate ATP synthesis from this substrate. These findings have important and far reaching implications concerning bacterial membrane energetics. Much work yet remains to be done in this area although we can now safely conclude that the cAMP-CRP complex is intimately involved in regulating membrane bioenergetics (7).

6. Role of cAMP-CRP in construction and function of the *E. coli* outer membrane and periplasmic proteins. The above discussed transport and energetic functions are believed to be associated primarily with the inner membrane - that is with the cytoplasmic membrane of the Gram negative envelope which includes an outer unit membrane as well as an inner membrane. Having shown that cAMP-CRP regulates inner membrane activities we sought to determine if it was also involved in regulating periplasmic and outer membrane proteins as well. To answer this question we analyzed some of the enzymes of the periplasmic region and the proteins of the outer membrane by SDS-gel electrophoresis.

Parental and mutant cells were subjected to mild osmotic shock sufficient to extract periplasmic proteins as described in the literature. Periplasmic acid and alkaline phosphatase but not cyclic phosphodiesterase were found to be reduced by approximately 50% by the cya and crp strains. This data showed that this portion of the *E. coli* envelope was influenced by cAMP-CRP regulation. Analyses of outer membrane proteins extracted by the Schnaitman procedure and separated by SDS-acrylamide electrophoresis showed that this region was also under cAMP-CRP control. One protein was almost totally

absent in the mutant cells. This protein has now been identified as the T_6 phage binding protein (3,12,13). During the course of these studies a novel technique was discovered for isolating the T_6 binding funding protein from outer membrane preparations (13). In this procedure a known concentration of T_6 phage is imbedded in the loading gel region of the acrylamide gel and the outer membrane sample added in the usual manner. After electrophoresis it can be shown that all proteins migrate through the gel except the T_6 -binding protein. The latter can be removed from the phage subsequently by increasing the electrophoretic current.

It has been reported by others that the T_6 binding protein is also the colicin K binding site, and that this site has some type of regulator function in controlling membrane ATP synthesis via electron transport activities. All proteins known to be regulated by cAMP-CRP have some involvement in the organism's carbon and energy metabolism. The T_6 binding protein seemed to be an exception to this rule. However, if it is indeed involved in the cell's bioenergetic function this principle still holds. We are obviously interested in carrying out further work with this particular outer membrane protein.

7. Fatty acid content under cAMP-CRP control. Fatty acid analyses showed that gga and gip mutant cells had lower levels of palmitoleic and cis-vaccenic, and higher levels of cis-9,10 methylene hexadecanoic acid than did the wild-type cells (5). It is not known what generalized effects these alterations may have on membrane organization, fluidity, etc., but it can be assumed that they do play some role in the organism's membrane functions.

8. Genetic suppression of the cAMP requirement. During our work with cya strains (E. coli strain C-57 in particular) it was observed that they tended to be unstable. We discovered that this instability was not the result of reversion back to the wild-type state, but rather, to the emergence of a new type of mutation. This new mutation was subsequently found to be a suppressor mutation for the cAMP requirement in this organism. These mutants have now been obtained from all cya strains available including a cya detection strain. They all retain the cya character but regain wild-type ability to grow on all substrates without need for endogenously produced or exogenously added cAMP. Not only do they no longer need cAMP, they are in fact inhibited by even low concentration of cAMP during growth on certain substrates. This inhibition was found to be the result of the following sequence of events: 1) in the presence of cAMP the suppressor mutants synthesize higher than wild-type levels of certain transport systems (e.g., xylose or β -galactoside transport); 2) high uptake rates increase the intracellular catabolite pools, including presumably dehydroxyacetone phosphate (DHAP), which is normally produced in only small amounts and which is the substrate for methylglyoxal (MG) synthase; 3) MG synthase levels increase approximately 2-5 fold during growth in the presence of cAMP, but the levels of MG dissimilating enzymes, glyoxalase I and D-lactate dehydrogenase, do not vary from wild-type levels. As a result of these cAMP-induced changes in these cells MG accumulates to a level of approximately 0.40 mM which is toxic to the cells and further growth ceases (10).

BEST AVAILABLE COPY

These suppressor mutants, designated csn mutations (cAMP suppressor mutations) have been studied genetically and found to occur in the crp region (8). It is thus proposed that the csn mutation causes synthesis of an altered CRP which functions in promoting initiation without modification by cAMP. We are currently attempting to analyze the CRP from these suppressor strains and characterize the changes brought about by the suppressor mutation.

9. Transport studies using the csn mutants. The csn mutants have a number of altered transport properties that are interesting and currently under investigation. Another interesting property which is perhaps related to transport activities is their ability to show a typical catabolite repression response to β -galactosidase synthesis despite the fact that they lack adenylate cyclase activity. Inhibition of the latter is thought to be the basis for catabolite repression. One can induce high rates of β -galactosidase syntheses in the csn mutants and then repress this synthesis by addition of glucose. This repression in turn can be completely overturned by addition of cAMP. Since adenylate cyclase can't be involved, this phenomenon is not true catabolite repression per se or the generally accepted mechanism explaining catabolite repression is incorrect. Work along these lines is also continuing.

10. Progress resume. In my opinion the work conducted under this contract was exceedingly productive. Clear insights were developed as to the role of cAMP-CRP as a regulator of key membrane activities in E. coli. Major transport and bioenergetic functions of the E. coli membrane system are regulated by this complex at the

genetic level. These studies were also productive in raising new issues and questions that allow us to probe deeper into the intricate biochemical processes that reside in biomembranes and which are of extreme importance to cell growth and survival.

III. Publication List and Bibliography for Final Report

A. Published Papers and Abstracts

1. Broman, R. L., D. White, and W. J. Dobrogosz. 1974.
Stimulation of cytochrome synthesis in Escherichia coli
by cyclic AMP. Arch. Biochem. Biophys. 162:595-601.
2. Ezzell, J. W., and W. J. Dobrogosz. 1975. Regulation of
substrate transport in Escherichia coli by cyclic AMP.
Abst. Ann. Meet., Am. Soc. Microbiol., p. 90.
3. Tseng, Y. H., S. S. Dills, and W. J. Dobrogosz. 1975.
Regulation of the outer membrane components of Escherichia
coli by cyclic AMP. Abst. Ann. Meet., Am. Soc. Microbiol.,
p. 117.
4. Ezzell, J. W., and W. J. Dobrogosz. 1975. Altered hexose
transport and salt sensitivity in cyclic adenosine 3',5'-
monophosphate deficient Escherichia coli. J. Bacteriol.
124:815-824.
5. Dallas, W. S., Y. H. Tseng, and W. J. Dobrogosz. 1976.
Regulation of membrane functions and fatty acid composition
in Escherichia coli by cyclic AMP receptor protein. Arch.
Biochem. Biophys. 175:295-302.
6. Dills, S. S., and W. J. Dobrogosz. 1977. Cyclic AMP
regulation of membrane energetics in Escherichia coli.
Abst. Ann. Meet., Am. Soc. Microbiol., p. 188.
7. Dills, S. S., and W. J. Dobrogosz. 1977. Cyclic AMP
regulation of membrane energetics in Escherichia coli.
J. Bacteriol. (In press, Sept. 1977)

8. Melton, T., L. Kimbrell, and W. J. Dobrogosz. 1977. The genetic and physiological characterization of a novel suppressor mutation in adenylate cyclase deficient mutants of Escherichia coli. Abst. Ann. Meet., Am. Soc. Microbiol., p. 188.

B. Paper Submitted for Publication

9. Ezzell, J. W., and W. J. Dobrogosz. 1977. Cyclic AMP regulation of the hexose phosphate transport system in Escherichia coli. J. Bacteriol. (Submitted for publication, July 1977).

C. Manuscripts in Preparation

10. Tseng, Y. H., T. Melton, and W. J. Dobrogosz. 1977-78. A new suppressor mutation for the cyclic AMP requirement in Escherichia coli. (To be submitted to J. Bacteriol. in Sept. 1977).
11. Melton, T., L. Kimbrell, and W. J. Dobrogosz. 1977-78. Genetic analyses of a suppressor mutation for the cyclic AMP requirement in Escherichia coli. (To be submitted to J. Bacteriol. ca October 1977).
12. Alderman, E., S. S. Dills, T. Melton, and W. J. Dobrogosz. 1977-78. Effect of cyclic AMP on the T₆ phage binding protein in Escherichia coli. (To be submitted to J. Bacteriol. ca Nov. 1977).
13. Alderman, E., and W. J. Dobrogosz. 1977-78. A new procedure for analyzing T₆ phage binding protein Escherichia coli. (To be submitted to Anal. Biochem. ca Nov. 1977).

14. Betancourt, D., T. Melton, and W. J. Dobrogosz. 1977-78.
Studies on PTS and cAMP-CRP double mutants of Salmonella
typhimurium. (To be submitted to J. Bacteriol., ca
Dec. 1977).
15. Sherba, D., Y. H. Tseng, and W. J. Dobrogosz. 1977-78.
Catabolite repression of the lac system in a cya mutant of
Escherichia coli. (To be submitted to Biochem. Biophys.
Res. Commun., ca Dec. 1977).

IV. Personnel Employed on Projects

1. Dr. Y. H. Tseng was supported as a pre-doctoral student and then as a postdoctoral student as indicated in earlier communications. Dr. Tseng is now on the faculty of the National Chung Hsing University, Taichung, Taiwan, Republic of China.
2. Dr. Thoyd Melton was employed as a postdoctoral student for the last 1 1/2 years of the project. Dr. Melton is now a member of the faculty of the Microbiology Department at the North Carolina State University.