United States Golf Association Green Section Research Proposal
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Genetic Basis of Biological Control in a Bacterium Antagonistic to Turfgrass Pathogens

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EXECUTIVE SUMMARY

The purpose of this project on *E. cloacae* genetics is to determine the array of bacterial traits responsible for biological control activity in bacteria. Our focus has been on *Pythium*-incited diseases of creeping bentgass, but we believe our studies will have broad applicability to other bacterium-pathogen interactions. The objectives of our studies are to: 1)identify and clone DNA sequences that encode pathogen-suppressive properties in *E. cloacae* strain EcCT-501, 2) determine the nucleotide sequence of *E. cloacae* DNA encoding pathogen-suppressive properties and tentatively establish a function for the gene product, and 3) evaluate, in field studies, the expression of the biocontrol-related gene, *psp1*, under typical turfgrass management conditions. Our studies in 1994 focussed primarily on objective 2. Prior to the initiation of the work, we had isolated mutant V58 which was unable to suppress *Pythium ultimum* seed rot of cucumber. We were able to verify that whereas the wild-type strain, EcCT-501, was an effective biological control agent of *Pythium graminicola* on creeping bentgrass, mutant V58 was an ineffective biological contol agent against this pathogen. Our work subsequently was focussed on the molecular and physiological characterization of mutant V58.

From complementational analysis, we have demonstrated that by rescuing the disrupted gene from wild-type cosmid library and mobilizing it into the mutant strain V58, we could effectively restore all of the biological phenotype of the wild-type strain. Using conventional molecular techniques, we have been able to isolate and sequence portions of the disrupted gene in mutant V58. From sequence analyses, we have discovered that the gene shares high homologies, both at the nucleotide and amino acid levels, with malate dehydrogenase from both *E. coli* and *Salmonella typhymurium*. We performed a series of enzyme assays to verify that the gene we had cloned was actually a malate dehydrogenase (Mdh) gene. Clearly, the wild-type EcCT-501 possessed high levels of Mdh activity. This activity was totally absent in mutant V58. Furthermore, upon complementation with the putative mdh gene, Mdh activity was restored. Therefore, we feel confident that we have discovered a malate dehydrogenase gene with a major influence on the biological control of *P. graminicola* on creeping bentgrass and *P. ultimum* on cucumber.

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Project Title: Genetic Basis of Biological Control in a Bacterium Antagonistic to Turfgrass Pathogens

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The primary goals of our research program are to identify genes in soilborne bacteria that regulate biological control processes and to study their expression in turfgrass ecosystems. The primary objectives outlined in our original proposal were three:

1) identify and clone DNA sequences that encode pathogen-suppressive properties in *E. cloacae* strain EcCT-501.

2) determine the nucleotide sequence of *E. cloacae* DNA encoding pathogen-suppressive properties and tentatively establish a function for the gene product.

3) evaluate, in field studies, the expression of the biocontrol-related gene, *psp1*, under typical turfgrass management conditions.

Progress in 1994

In generating mutants, we have used an insertional mutation approach with the transposon mini-Tn*5*/*phoA*. This was chosen as the mutagen not only for the nearly random genomic insertion that is a key property of the Tn*5* system, but for the *phoA* (bacterial alkaline phosphatase) reporter gene portion of the construct that enables us to screen for mutations which are expressed as changes in the proteins on the cell surface of the bacteria. Our mutational approach facilitated a search for genes in *E. cloacae* that both affect the bacterial cell surface and play a direct role in bacteria's interaction with their surrounding environment.

The property of bacterial alkaline phosphatase that was taken advantage of in constructing tranposons with this gene as a reporter is that the enzyme can only be expressed if it is transported through the bacterial plasma membrane. Topologically, therefore, this gene's activity can only be detected if the protein resides in or on the outer surface of the plasma membrane, in the periplasm, or in the cell wall or outer membrane of enterobacteria. The *phoA* reporter, like any translational reporter gene, lacks both the promoter and translation initiation signals normally found in genes. Therefore, its expression is dependent on a promoter and translation regulation of a gene in the host cell. The only way, therefore, that *phoA* will be expressed is for the reporter/tranposon to be inserted in-frame and downstream from the translation start codon of a resident gene which is itself normally transported through the plasma membrane.

We have chosen to bias our study toward discovering traits that are expressed in a particular topological orientation, and study those which when mutated alter the overall quality of the bacteria's ability to protect turfgrass plants from *Pythium* species

and other soilborne pathogens. Thus, our approach has not been to screen bacteria for some specific property whose role in biological control is unknown. Instead, we have used the mini-Tn*5*/*phoA* transposon as a mutagen to first seek mutations in genes that are expressed at the cell's interface with its surrounding environment, the plant, and other microbes, and then to screen that subset of mutants for alterations in overall biological control. As is described below, this approach, in conjunction with other research in our laboratory, has led to some potentially puzzling findings, and a hypothesis for an unanticipated role in biological control for a Krebs cycle enzyme.

The work that has been accomplished this year has been primarily under objective 2. By the time this grant was initiated, we had generated a library of mini-Tn*5*/*phoA* mutants of *E. cloacae*, and identified several mutant strains that displayed reduced biological control effectiveness. As noted in our original proposal, we had initially focused on one strain in particular, designated V58, which exhibited a marked loss of biological control activity on two different test systems in bioassays, namely turfgrass and cucumber. Because mutant V58 exhibited such drastic changes in biological control ability, we have focused in the intervening months primarily on characterizing V58 from a molecular and physiological standpoint.

Results

Initial isolation of mutant V58.

From our mutant library, *phoA*+ mutants were detected by the appearance of blue colonies on selective agar media containing the compound X-phos (5-bromo-4-chloro-3-indolyl phosphate) which is cleaved by alkaline phosphate to generate a blue product. The mutant V58, which produced a blue colony on such media, exhibited marked loss of ability to suppress *Pythium* damping off on both cucumber (*P. ultimum* isolate P4) and creeping bentgrass (*P. graminicola* isolate PRR-8) (Tables 1 and 2).

Marker rescue and complementation of insertionally-mutated V58 DNA.

The mutant gene was isolated from strain V58 by marker rescue. A DNA hybridization probe was made from a restriction fragment of the mini-tn*5*/*phoA* transposon. Hybridization of this probe to genomic DNA of V58 revealed the presence of a single copy of the transposon, which was assumed to reside within a gene whose insertionally-interrupted expression had led to the loss of biological control by V58. The tranposon probe hybridized to a single *Kpn*1 fragment about 18 kb long. A *Kpn*I library of V58 DNA was prepared in the pUC 19 vector. The vector carried ampicillin resistance, and the desired fragment was the only V58 genomic fragment that was expected to carry the kanamycin resistance gene (from the tranposon), so the V58 genomic library was screened on media amended with both antibiotics. Nine out of the ten resulting clones that were subsequently detected and subjected to Southern hybridization contained an 18 kb *Kpn*1 fragment whose restriction digest pattern was identical to hybridized genomic fragment. The resulting plasmid was named pV58K.

A crude restriction map of plasmid pV58K was constructed, a probe was prepared from the border between the transposon and the mutant gene. This probe was used in colony hybridizations to a cosmid library of EcCT-501 DNA (Loper *et al.*, 1993?). Three identical cosmids were detected, each containing approximately 28 kb of EcCT-501 DNA, including an approximately 16-kb *Kpn*I fragment. The patterns of the cosmid's restriction digest, using six different restriction enzymes, and hybridizational

pattern with probe 2, were consistent with those of the insert of pV58K, less the transposon insert. (Fig. 1).

The wild-type cosmid (cos 2.2) and the cosmid vector pLAFR3 were separately transferred to mutant V58 by triparental mating, and recovered by selection on minimal media amended with kanamycin and tetracycline, verifying that the resulting transconjugants, V58Cos and V58LAF, carried both the transposon and a Tc-resistant cosmid, as expected.

The new isolates, along with EcCT-501 and V58, were subjected in parallel to the cucumber and creeping bentgrass bioassay. In multiple tests, EcCT-501 and V58Cos were equivalent in their suppression of *Pythium* pathogenicity on cucumber, while V58 and V58LAF were both ineffective (Table 2). Similar results were obtained in tests of EcCT-501 and V58 on bentgrass (V58Cos and V58LAF were not tested on bentgrass).

The gene carried on the cosmid, which complemented the biological control defect of V58, was provisionally named *psp1*, for Pythium suppressive.

Subcloning and Sequence analysis

Sequencing of *psp1* is a critical part of this project, both for identifying the putative function of the gene and in order to make constructs that facilitate experiments on the expression and regulation of the gene's role in biological control. Few convenient restriction sites are available for subcloning in the wild type cosmid. Subclones of pV58K were constructed, therefore, because several convenient restriction sites for subcloning are available in the tranpsoson itself, and sequencing from the transposon outwards into the surrounding gene, in both directions, allows the most direct method for assuring that one is sequencing a gene which is otherwise unidentified.

Sequencing has been performed with the use of oligonucleotide primers generated from known sections of previously sequenced portions of subclones, beginning with the *phoA* and pUC portions of the subclones. Sequencing was done on an automated sequencer at the Cornell University Biotechnology Center. The upstream portion of *psp1* has now been sequenced from a 5.2 kb HindIII subclone of pV58K (Fig. 2). That the sequenced region is the upstream portion of *psp1* was verified by a) the use of a primer homologous to the antisense strand of the reporter gene *phoA*, and 2) location of the gene/reporter fusion site on the resulting sense strand of the sequence.

Data base analysis of the sequenced upstream portion of *psp1* demonstrated that the inferred amino acid sequence of the gene was identical to that of malate dehydrogenase from both *Escherichia coli* and *Salmonella typhimurium*. With this close a match, of course, there is immediately the suspicion that the cloning of such a gene represents an artifact of cloning processes, and that the gene is not really from *E. cloacae*. However, the nucleotide sequence of the *E. cloacae* gene diverges from that of the other two genes by 20%, and the divergences are non-identical themselves, so we are confident that the identification of *psp1* as malate dehydrogenase is valid, and that *psp1* is from *E. cloacae*.

Despite many attempts, however, the downstream portion of pV58K has not been subcloned or sequenced. Repeated subcloning experiments, using different approaches, have been frustrated due to unspecified technical or biological complications with the particular sequence. Regardless of the specific fragment, vector, or orientation of

vector relative to fragment, the region has proven unrecoverable in the subclone, despite the entire parent clone and the cosmid being easily recovered, and despite the upstream fragment being easily recovered in parallel experiments. We suspect that part of the problem lies with the biological or genetic properties of the large fragment in the downstream region that must be cloned using known restriction sites. In the ensuing months, new approaches using PCR (polymerase chain reaction) strategies will be attempted. It has been possible to transfer the entire *E. coli* malate dehydrogenase (Mdh) gene between strains of that organism, so the same should be possible for the *E. cloacae* gene. It may be possible to generate a PCR clone of the downstream portion of the *E. cloacae* Mdh gene by using primers homologous to the *E. coli* Mdh gene.

Mutant V58 Mdh Activity and growth on Single-source Media.

Mutant strain V58 lacks functioning malate dehydrogenase, while the complemented mutant (V58Cos) has regained Mdh activity (Fig. 3). Cell extracts of EcCT-501function of malate dehydrogenase in wild type and V58 cells were examined to determine whether the transposon residing in the *E. cloacae* Mdh gene actually caused a loss of Mdh activity. V58 has no enzyme activity whatsoever, which is consistent with the position of the transposon within the coding region of the gene, and with the existence of only one copy of Mdh in enteric bacteria. The restoration of activity in V58Cos suggests that the enzyme functions in *trans* configuration relative to the genome, or has reintegrated into the genome.

Strain V58, EcCT-501, and V58Cos were all grown on minimal medium amended with either glucose or malic acid as single carbon sources (50 mM). As shown in Figure 4, these strains all grew at nearly equivalent rates on glucose, with V58 somewhat slower than either wild type or complemented mutant. On malate, however, the results were much different. Overall, all strains' growth on malate was slower, and with a slightly longer lag before log phase, than the same strains on glucose (Fig. 4). However, it is notable that V58 grew very slowly on malate, compared to either the parental strain or the complemented mutant. It is also notable that these assays were monitored closely for approximately seven hours. EcCT-501, V58, and V58Cos all had approximately equivalent cell densities after overnight growth on these media, at stationary phase, with optical densities of about 2.0 A_{600} units, so the limitations on V58 growth on malate are in the rate of growth and, presumably, the rate at which this strain can compensate with other enzyme systems for the lack of Mdh. Subsequent experiments of this type will use cultures that have been grown for a single passage on the single-carbon source media before initializing the growth rate measurements, to determine whether pre-adaptation to malate makes a difference in the growth rates of these strains.

An intriguing contrast to V58 is presented by the data of strain 21.1 and 21.1Cos (Figs. 3B and 4). These are a mutant and complemented mutant isolated in a separate study by a graduate student in our laboratory, K. van Dijk. Strain 21.1 is a mutant which was generated with the same mini-Tn*5*/*phoA* protocol but does not express *phoA* on X-phos medium. It grows poorly on linoleic acid, which has been shown in our laboratory to be to one of the primary germination stimulants of *P. ultimum* . Interestingly, it fails to grow and, apparently, to adapt to the presence of malic acid, even though it possesses Mdh activity.

Interpretation and Plans for Subsequent experiments.

The experiments on growth rates on single carbon sources, and enzymatic assays are consistent with the identity of *psp1* as *E. cloacae* malate dehydrogenase. Mdh is a single copy gene in *E. coli*, and therefore probably also in *E. cloacae* because of the very high degree of relatedness between the two species. Mutation of Mdh should lead to complete loss of Mdh activity, as seen for V58.

Loss of Mdh represents the loss of a key enzyme in the tricarboxylic acid, or Krebs, cycle. However, growth on complex media should not be seriously compromised because bacteria have anaplerotic pathways for replenishing Krebs cycle intermediates when they are unavailable or siphoned off by anabolic pathways. On single-carbon defined media, however, the situation is more difficult to interpret. It is demonstrated that malate can support (relatively) slow growth of an Mdh mutant, presumably through the activation of anaplerotic pathways also. As expected, the adaptation to malate as sole carbon source, and the overall rate of growth, is slower than for wild type cells with functioning malate dehydrogenase enzyme. So we conclude that the strain V58 represents a single mutation in the gene for malate dehydrogenase.

Why should malate dehydrogenase mutation lead to a loss of biological control in this mutant, and why should this mutation have been detected by expression of alkaline phosphatase? The answer to the second of these questions is straightforward if Mdh is a plasma membrane-bound enzyme. The certainty of this seems to have been established. The results of crossed immuno-electrophoresis experiments and activity stains of native enzyme that Mdh was present in the plasma membrane fraction of *E. coli* cells (Smyth *et al.*, 1978. The orientation of the enzyme relative to the cytoplasm was not described however. A few anecdotal comments in Gottschalk (1979) and other sources suggest that Mdh is the receptor for malic acid. However, if Mdh were a receptor for malic acid, then from the data on growth of V58 on malate, Mdh can not be the only way that malate can enter the bacterial cell.

Our current hypothesis for the involvement of malate dehydrogenase in biological control has been arrived at through other work in our laboratory pertaining to the nature of the germination stimulants in seed and root exudates that trigger germination of *Pythium* propagules. We have demonstrated that long-chain fatty acids (LCFA) are a primary germination stimulant, and that at least one way that *E. cloacae* interferes with *Pythium* response to seeds and roots is to metabolize these germination stimulants and prevent the propagules from germinating. This process seems to be dependent on uptake and metabolism of the fatty acids in germination stimulants, accomplished through a specific receptor, activation of the fatty acids with coenzyme A, and subsequent β-oxidation of the activated acyl-CoA molecules. In *E. coli*, the terminal pathway of this process is the dicarboxylic acid cycle. This involves the fusion of acetyl-CoA units with glyoxylate to form malate, which must be converted to oxaloacetic acid by Mdh, the beginning of a cycle that eventually generates pyruvate again for entry into the Krebs cycle (Fig. 5). Lacking the Mdh would presumably further slow the process of uptake and metabolism of the LCFA, inhibiting bacterial metabolism of germination stimulants and permitting propagule germination to ensue.

This hypothesis is consistent with our observations of bacterial metabolism of germination stimulant in a variety of seed exudates including creeping bentgrass and perennial ryegrass. However, the *P. graminicola* inoculum used in our bioassays is primarily mycelium, and the nature of the interactions among the *E. cloacae*, *P. graminicola*, and creeping bentgrass are virtually unknown at this time. We anticipate that further study of *psp1* and the generation of other mutants will add significant

insight to our understanding of these interactions, and are a major priority of the work to be pursued with support from the current USGA grant in the coming year.

References

Gottschalk, Gerhard, 1979. *Bacterial Metabolism.* New York: Springer-Verlag.

Smyth, C. J., Siegel, J., Salton, M. R. J., and Owen, P., 1978. Immunochemical analysis of inner and outer membranes of *Escherichia coli* by crossed immuno-electrophoresis. J. Bacteriol. 133: 307-319.

Owen, P., and Salton, M. R. J., 1975. Antigenic and enzymatic architecture of *Micrococcus lysodeikticus* membranes established by crossed immuno-electrophoresis.

Figures

Table 2. Comparative biological control activity of Tn*5*/*phoA* mutants of *Enterobacter cloacae* with the wild-type strain EcCT-501 and strains of *Escherichia coli*

	Cucumber Seedling Emergence (%)				
Bacterial Strain	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
EcCT-501 (Wild-Type)	66.7	100.0	83.3	100.0	66.7
501/V-58	16.7*	33.3*	0.0*	0.0*	0.0*
501/11-84	-^	-	33.3*	66.7	66.7
501/17-88	-	-	33.3*	33.3*	50.0*
E. coli HB101	-	-	-	0.0*	0.0*
E. coli DH5_	-	-	-	-	16.7*

^ - = strain was not tested.

Means followed by (*) are significantly different (P=0.05) from those of strain EcCT-501 according to T-tests.

Table 1. Comparative biological control activity of Tn*5*/*phoA* mutants of *Enterobacter cloacae* with wild-type strain EcCT-501 and strains of *Escherichia coli*.

bacterial amendment:	none (pathogen only)	EcCT-501	V58	none (germin'n control)
avg rating (five reps.):	4	1.6	4	1

Table 2 Comparison of biological control activity of EcCT-501 with mutant V58 against *p. graminicola* isolate PRR-8 on creeping bentgrass. Ratings of grass seedling stands ranged from 1 (full healthy stand)to 5 (10% germinated)

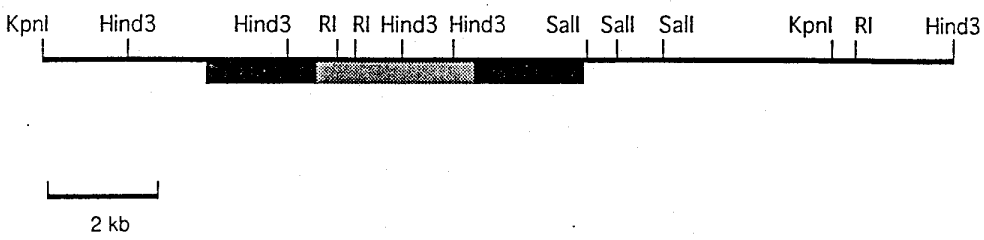
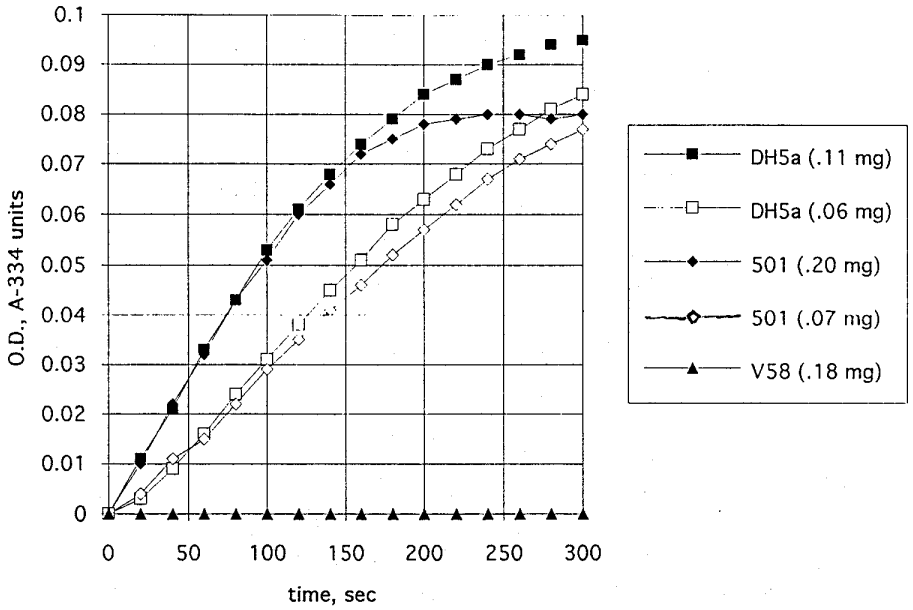


Fig. 1. Map of plasmid pV58/K, isolated from mutant V58. Approximate locations of the transposon (light shading) and inferred location of left and right ends of the *psp1* gene (dark shading) are indicated. Selected restriction sites are shown.

```
5' (nt)  AGTCGCGTGACCNCAAATTCTTTAGCTACGAAAATTGTAATTATTTACTTGCTGAATTAT
      1 ---------+---------+---------+---------+---------+---------+ 60
  (aa)  S  R  V  T  ?  N  S  L  A  T  K  I  V  I  I  Y  L  L  N  Y  -

        GGTCCCCGCAACGGATTTACAGATACTTAAGTTAACCATAATAAGGAGTTTAGGATGAAA
     61 ---------+---------+---------+---------+---------+---------+ 120
        G  P  R  N  G  F  T  D  T  *  V  N  H  N  K  E  F  R  M  K  -

        GTCGCAGTCCTCGGCGCTGCTGGTGGTATCGGCCAGGCGCTTGCCCTACTACTGAAAACC
    121 ---------+---------+---------+---------+---------+---------+ 180
        V  A  V  L  G  A  A  G  G  I  G  Q  A  L  A  L  L  L  K  T  -

        CAACTGCCTTCAGGCTCAGAACTCTCCCTGTACGATATTGCTCCGGTAACCCCAGGTGTG
    181 ---------+---------+---------+---------+---------+---------+ 240
        Q  L  P  S  G  S  E  L  S  L  Y  D  I  A  P  V  T  P  G  V  -

        GCGGTTGACCCTGACTCTTGTACACAAGTAGCGTCCTGGACNGANCTT       3'
    241 ---------+---------+---------+---------+-------- 288

        A  V  D
```

Fig. 2. Sequence from upstream portion of *psp1* gene from *E. cloacae* isolate EcCT-501. The 249 nucleotides of the gene upstream from the *phoA* reporter gene in mutant V58 are displayed. (A total of approx. 600 nt have been sequenced, contiguous with this portion). Deduced amino acid sequence is shown underlined, with the initiator methionyl residue indicated in bold type. Putative Shine-Dalgarno ribosome binding site for the mRNA is indicated in italics. Nucleotide 250 is the first nucleotide of the alkaline phosphatase reported sequence contiguous with the *psp1* gene in the mutant strain.

A.



B.

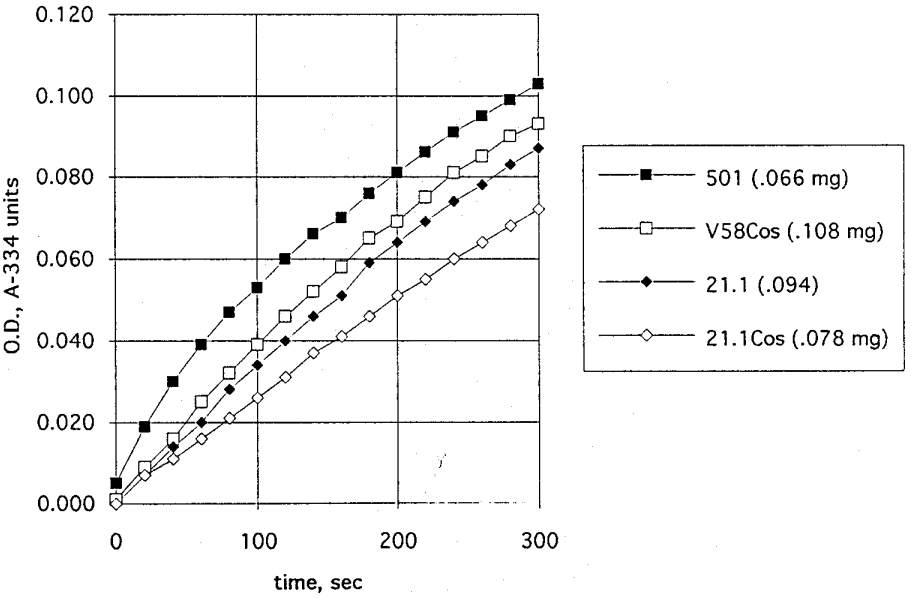


Fig3. Malate dehydrogenase activity of selected *E. cloacae* strains. Cells were grown on rich medium, then disrupted by sonication. Extracts were clarified by centrifugation and assayed in a reaction mixture consisting of 50 mM Tris buffer (pH 7.5), 50 mM malic acid, 3 mM NAD. Reaction was monitored spectrophotometrically for evolution of reduced NAD at a wavelength of 334 nm. Amount of total cell extract protein used in each assay is shown in parenthesis. **A.** Parental strain EcCT-501, *E. coli* strain DH5α, and mutant V58. **B.** Strain EcCT-501, along with the complemented mutant V58Cos, and another mutant, 21.1 and the same strain carrying the complementing cosmid, 21.1Cos.

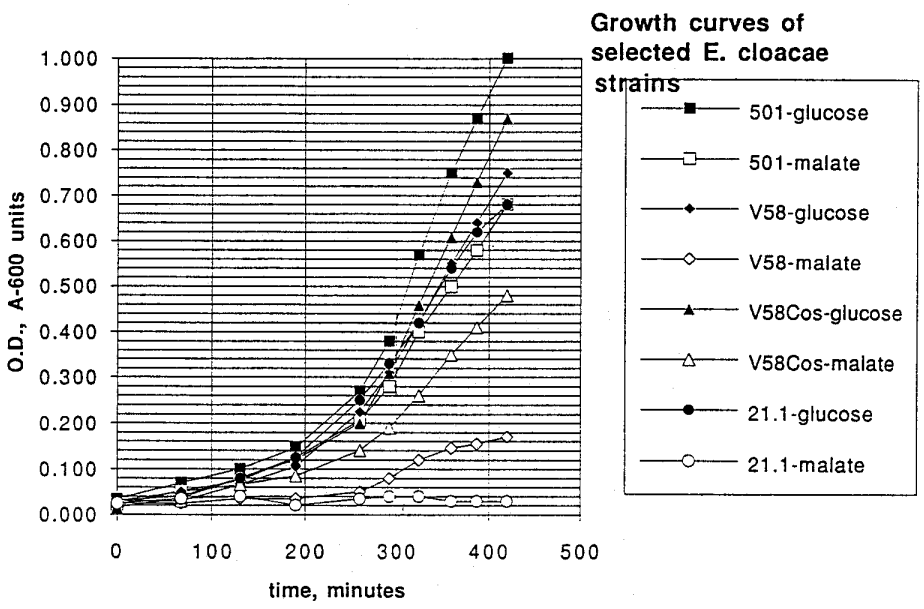


Fig. 4. Growth of selected *E. cloacae* strains on single carbon sources. Growth medium consisted of a minimal mineral salts medium (M56) supplemented with 50 mM of either glucose of malic acid.

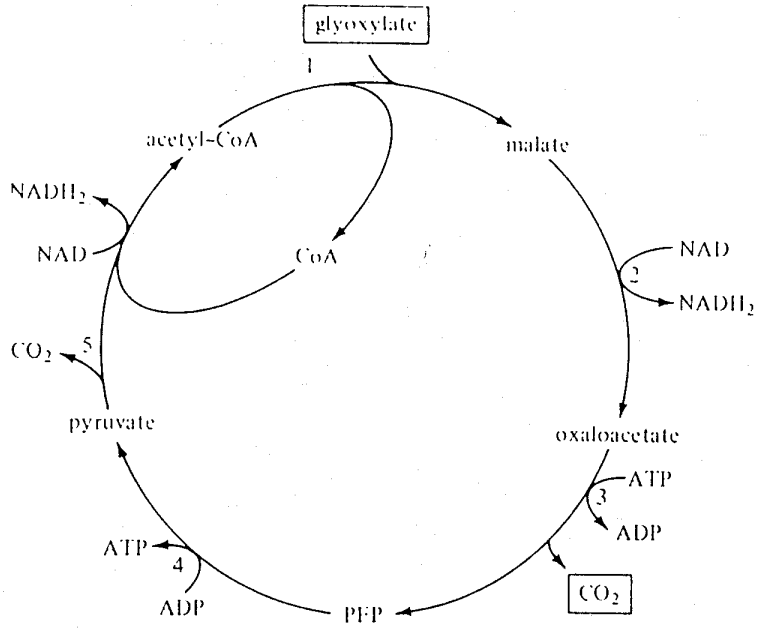


Fig 5 Dicarboxylic acid cycle. Enzymes are: 1, malate synthase; 2, malate dehydrogenase; 3, PEP carboxykinase; 4, pyruvate kinase; 5, pyruvate dehydrogenase. (Adapted from Gottschalk, 1979)