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PROGRESS REPORT

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Project Title: **Genetics and Molecular Biology of Hydrogen Metabolism in Sulfate Reducing Bacteria**

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DISCLAIMER

SUMMARY

The work proposed to be accomplished in the previous funding period was to develop a procedure for genetic exchange based on conjugation mediated by broad host-range plasmids. Such a system has recently been identified that employs IncQ group plasmids and a Desulfovibrio desulfuricans G100A derivative as recipient (described in the Progress Report). Now a library of the DNA from this bacterium is being prepared in an IncQ cosmid vector, pJRD215. During the search for conjugation, we also identified a defective bacteriophage capable of generalized transduction of 13.5 kb fragments of chromosomal DNA between mutants of Desulfovibrio desulfuricans ATCC strain 27774 (attached PNAS paper). Some of the factors influencing the production and transduction by this defective phage have been investigated (Progress Report).

A curious observation was made concerning the response of colonies of these sulfate-reducing bacteria upon exposure to air. All the cells of a colony do not die. Some survive, most likely by producing sulfide at a rate sufficient to provide an anaerobic environment. Dramatic colony morphological changes occur and these have been documented by scanning and transmission electron miscroscopy (preprint attached).

Finally a small endogenous plasmid has been isolated from D. desulfuricans G100A (Progress Report). It has been stably subcloned into a sequencing vector, pTZ18U, and nested deletions of this plasmid are being prepared. This plasmid may be useful for the development of a shuttle cloning vector that could be used in more diverse Desulfovibrio.

Many of the techniques now to be used in the mutant analysis of hydrogenase genes in the sulfate-reducing bacteria have been successfully applied in an analysis of hydrogenase functions of Rhodobacter capsulatus. This work is described in the attached Journal of Bacteriology paper.

Progress Report

I. Transduction

We have recently reported the identification of an apparently defective bacteriophage, Ddl, present in culture filtrates of D. desulfovibrio ATCC strain 27774 that is capable of mediating generalized transduction (Rapp and Wall, 1987). The parameters affecting the transduction process have been further investigated and the host range of Ddl explored.

Not surprisingly younger cultures were more efficient as recipients than were cultures in stationary phase. Because the phage is apparently always being produced by strain 27774, the transduction frequency could be increased 2-3 fold by removing the recipient's phage by centrifugation of the recipient culture prior to transduction.

If the packaging of transducing DNA in the donor is random and its integration and expression in the recipient a uniform probability, then all markers should be transduced with equal frequencies. This is in fact the case for the generalized transducing vector, Gene Transfer Agent (GTA), in Rhodobacter capsulatus. We observed that the drug resistant markers used in 27774 were not transferred at equal frequencies. Resistances to nalidixic acid and novobiocin were consistently transduced 5 to 10 fold more frequently than resistance to rifampicin or neomycin. Additional spontaneous mutations were isolated for each of the resistances that confirmed the allele independence of the effect. DNA packaging into phage particles could be non-random or the selection for transductants with antibiotics that interfere with gyrase might affect integration frequencies. Addition markers must be screened before a conclusion can be drawn concerning the nature of the packaging event.

In generalized transduction mediated by P1 in E. coli or P22 in Salmonella typhimurium, only 10% of the chromosomal DNA fragments transferred are stably integrated. This can be increased to almost 100% by a small dose of UV irradiation. Apparently the damaged DNA induces the production of RecA protein stimulating recombination. We therefore wanted to see if a small dose of UV could increase the transductants obtained with Ddl. If not, this would be taken as evidence that the transferred fragments are efficiently integrated in 27774. The results are shown in Fig. 1. These data confirm that no increase in transductants was obtained when Ddl was subjected to increasing UV irradiation (Δ). Also the rate of inactivation was similar to that of the

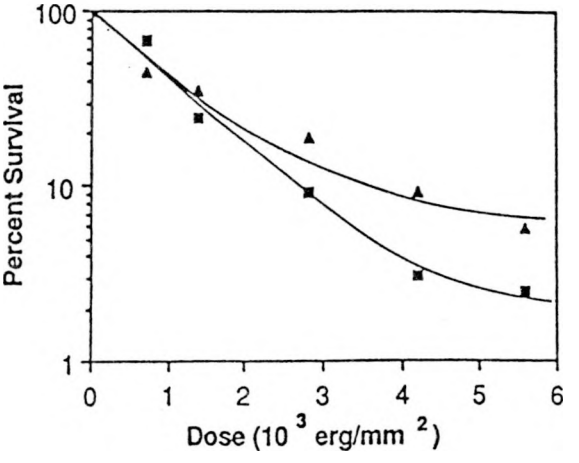

Fig. 1. UV inactivation of transducing ability of Ddl (■) and GTA (▲).

generalized transducing vector GTA from R. capsulatus. These rates are several orders of magnitude lower than for the inactivation of plaque forming ability of nondefective phage and are expected when the target site for inactivation of transduction is perhaps a few hundred bases.

An analysis of the frequency of double gene transfers gives an indication of the proportion of the cells in a population that is active as recipients in transduction. The calculated frequency of a Ddl induced double event is the product of the single event frequencies, e.g. 5×10^{-10} for Nov^R and Rif^R. The observed Ddl frequency of transduction of the two markers, Nov^R and Rif^R, to a single recipient was $< 1.1 \times 10^9$. We interpret this to mean that the total population of cells can be recipients for transduction.

Experiments were carried out to determine whether cells or phage might be limiting in our standard transduction assay. In that assay, donors are mid-to late-exponential cultures while recipients are early exponential cultures. Equal volumes of recipient cells and donor filtrate are mixed, allowed to incubate overnight, and aliquots plated with an appropriate selective antibiotic. When decreasing volumes of filtrate were added, it was observed that the number of transductants changed little until a rather large decrease was obtained (Fig. 2). Thus the phage exhibited saturation and at

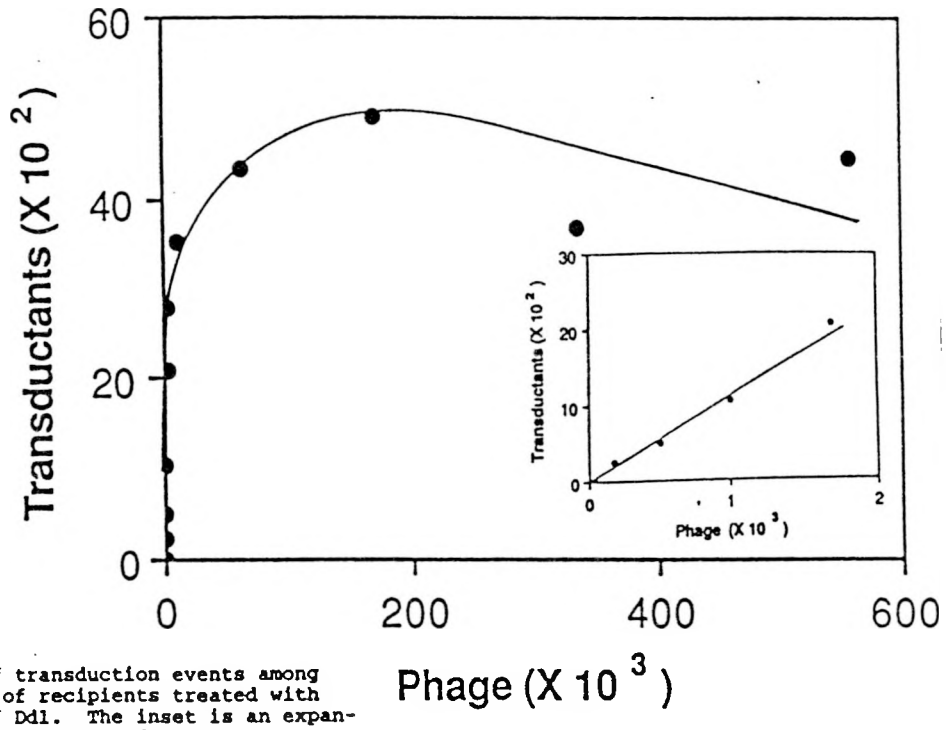

Fig. 2. Number of transduction events among a constant number of recipients treated with various amounts of Ddl. The inset is an expansion of the initial portion of the curve.

low dilutions a linear response of numbers of transductants per volume of input phage was obtained. Because cells rather than phage were limiting in a number of experiments, the transduction frequencies may have been underestimated.

Drug resistant derivatives of several Desulfovibrio strains were isolated to test for intrastrain gene transfer mediated by a transduction process. The strains and drugs used are shown in Table 1. No intrastrain transduction was observed. In addition no transfer to or from strain 27774 in crosses with any of these Desulfovibrio strains could be documented.

Table 1. Desulfovibrio species examined for transducing ability

Stain	Selected Marker
D. multispirans	Nal^R
D. gigas	Neo^R
D. desulfuricans Norway	Neo^R, Nal^R
D. desulfuricans G100A	Nal^R
D. desulfuricans ATCC 13541	Neo^R, Nal^R, Nov^R

A serious problem was manifest during these experiments. After a large series of tests, it was found that the batch of yeast extract used had a profound effect on the production of transducing phage. Batches from Difco were especially poor, while the Sigma Chemical Co. product was best. Variation from batch to batch is still a problem.

II. Conjugation

The most important observation made during the previous funding period was the conjugation of IncQ plasmids from E. coli to a nalidixic acid resistant derivative of D. desulfuricans strain G100A. This strain was made available to us (M. Odom, Dupont Chemical Co.) because it has rapid growth on defined medium (which strain 27774 does not) and a spontaneous Nal^R mutant, G200, was selected. The Q incompatibility group plasmid pKT230 (Fig. 3) conferring kanamycin resistance was mobilized with plasmid pRK2013 as helper.

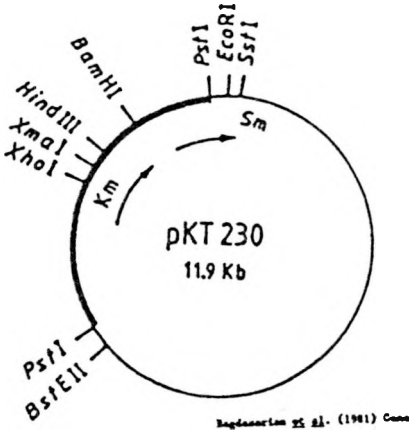

Fig. 3. Map of the IncQ plasmid pKT230. Bagdasarian et al., Gene 16:237, 1981.

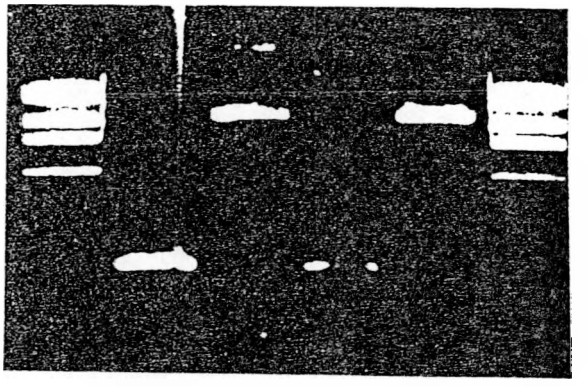

Fig. 4. Transfer of pKT230 into Desulfovibrio was confirmed by EcoRI restriction analysis of CsCl purified plasmid DNA from: B, the Desulfovibrio recipient; C, the E. coli donor; D, the exconjugant; E, a transformant obtained from the exconjugant plasmid; A and F, HindIII cut lambda.

Controls with pRK2013 alone (which also encodes kanamycin resistance) yield no exconjugants, confirming the supposition that pKT230 was the plasmid stabilized. Plasmid was prepared from the D. desulfuricans exconjugant (shown to be free of any contaminating E. coli donor). The plasmid was both visualized on agarose gels (Fig. 4) and used to transform E. coli strain DH5α to Kan^R. The Desulfovibrio recipient contained an endogenous plasmid of about

2.5 kb. After conjugation, both the native plasmid and pKT230 were present. The DH5α transformant clearly contained a plasmid of equal mobility to pKT230 and interestingly no plasmid the size of the Desulfovibrio endogenous plasmid.

The versatile Q-group cloning vector pJRD215 (Fig. 5) was obtained after the initial success with pKT230. This plasmid was also mobilized into G200 as

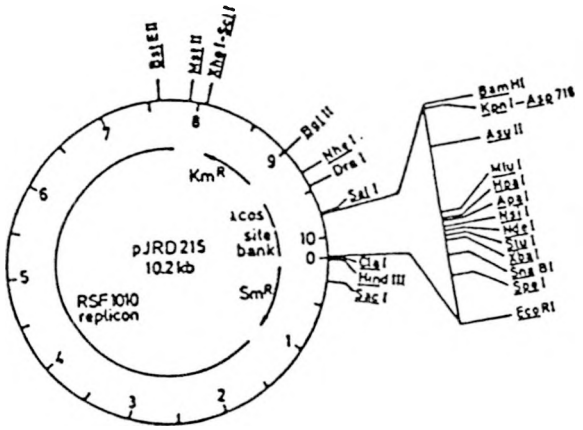

Fig. 5. Map of the IncQ plasmid pJRD215. Davison et al., Gene 51:275, 1987.

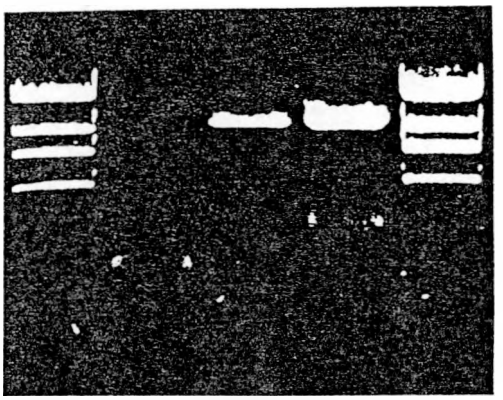

Fig. 6 Transfer of pJRD215 into Desulfovibrio was confirmed by EcoRI restriction analysis of CsCl purified plasmid DNA from: B, the Desulfovibrio recipient; C, the E. coli donor; D, a transformant obtained from the exconjugant plasmid; A and E, HindIII cut lambda.

confirmed by plasmid preparations (Fig. 6). Sufficient plasmid DNA was not obtained from the G200 exconjugant to visualize on the gel. However, that DNA was successfully used to transform DH5α to Kan^R and the plasmid from the transformant was the correct size for pJRD215.

Conjugation of other plasmids was also tested and it was observed that selection for expression of Kan^R frequently allowed conjugation to be detected. Thus a major limitation in previous attempts at conjugation is likely to have been the use of tetracycline and other markers as the selectable phenotypes.

Table 2. Conjugational transfer of plasmids of different incompatibility groups into Desulfovibrio desulfuricans.

Plasmid	Incompatibility Group	Selectable Marker	Average Frequency
pKT230	Q	Km^r	10^{-5}-10^{-5}
pJRD215	Q	Km^r	10^{-5}-10^{-5}
pRHP20.2d	P	Km^r	10^{-5}
pPH1J1	P	Gm^r	> 10^{-8}c
pSF6	W	Sp^r	< 10^{-8}d
pSUP2021	pMB1 replicon	Km^r	> 10^{-8}
pWB5a	P	Tc^r	< 10^{-8}
RFS1010	Q	Sm^r	< 10^{-8}

a Frequency is the number of exconjugants per donor cell.

The observation that a few kanamycin resistant colonies were obtained over the spontaneous frequency with pSUP2021 suggests that these exconjugants may harbor Tn5. This possibility is quite exciting for the development of transposon mutagenesis in this species and direct evidence for Tn5 sequences will be sought.

Already a collaborative effort is underway with Dr. Gerrit Voordouw from the University of Calgary. He first reported cloning the (Fe) hydrogenase from Desulfovibrio vulgaris into E. coli. Unfortunately although the polypeptides were made there, the enzyme was inactive. Dr. Voordouw would like to introduce the D. vulgaris genes into D. desulfuricans to see if expression and activity can be achieved. First he is probing the chromosomal DNA of strain G200 (prepared by us) to see if it possesses an (Fe) hydrogenase. He is also constructing the pJRD215 recombinant plasmid containing the D. vulgaris genes. We will carry out the conjugations and confirm the physical presence of the sequences. Dr. Voordouw will examine expression. This system may prove to be a useful intermediate for the functional expression of genes from other sulfate-reducing bacteria.

III. Endogenous Plasmid pBG1.

The isolation of the 2.4 kb endogenous plasmid from D. desulfuricans mutant G200 was a surprise. Of 16 strains of Desulfuricans previously examined by Dr. J.R. Postgate none had plasmids less than 40 kb and none could be isolated in useful quantities. The plasmid, pBG1, was digested with EcoRI and subcloned into pTZ18U to form pSC1 of 5.2 kb that is stably maintained in E. coli. The restriction map of pSC1 shown in Fig. 7 was generated by digestion with DdeI. pBG1, the native plasmid, is cut with DdeI as well.

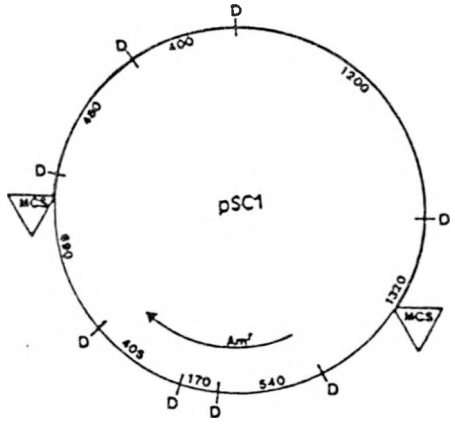

Fig. 7. DdeI (D) restriction map of the chimeric plasmid, pSC1, 5.2 kb. Thick line represents pBG1 sequences; thin line, pTZ18U.

This sensitivity to DdeI proves that D. desulfuricans strain G200 does not possess a modification system identical to that found in D. baculatus (Norway) [formerly D. desulfuricans (Norway)] from which DdeI is purified. This small plasmid may form the basis of a useful shuttle vector in species of Desulfovibrio.

IV. Transformation

Although attempts to transform D. desulfuricans strains have continued, they have been uniformly unsuccessful. With the knowledge that IncQ plasmids could be stably maintained and the Kan^R marker expressed in these bacteria, renewed efforts to observe transformation were initiated. Still no transformation could be documented. With the development of a phenotypically marked endogenous plasmid (proposed here), transformation will again be tried.

V. Electroporation

Electroporation has been shown to be a useful tool for the introduction of DNA into non-transformable Gram-negative bacteria. Because of the promise of this technique, we attempted a few preliminary experiments on a demonstration instrument. Obviously, the difficulties of performing these experiments with a strictly anaerobic organism reduce the probabilities for success. However, we monitored the response of the cells to short exposures of O_2 and obtained recoveries that should not have precluded observation of successful electroporation. Still experiments were performed with reasonable precautions regarding exposure to O_2. However, viable sulfate reducers were not recovered. Among the possibly problems, two are most prominent. First, the electroporation may have allowed the small amounts of O_2 present in the buffer to penetrate the cells, thus inactivating O_2-sensitive functions. Second is the possibility that the conductivity could not be readily controlled because of a matrix material associated with the bacterial cells. Figure 8 shows an electron micrograph of a D. desulfuricans cell prepared from

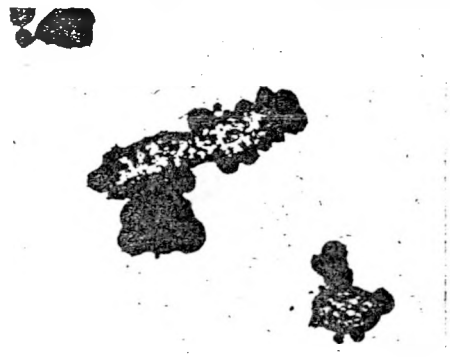

Fig. 8. Transmission electron micrograph of Desulfovibrio desulfuricans ATCC 27774 grown in liquid medium, lactate + sulfate as substrates.

a liquid culture grown in our standard medium for sulfate reduction. The black material surrounding the cells is not primarily polysaccharide since EPS is not visualized by staining with osmium tetroxide. However, osmium sulfide is a highly insoluble, electron dense material. When osmium was not used for staining the black material was not seen. Thus the sulfate-reducers may have the capability of maintaining a redox-active "overcoat" that is not removed by casual washing. Until this intriguing possibility is resolved, electroporation will not be seriously pursued.