Increased Fertility of Corynebacterium glutamicum Recipients in Intergeneric Matings with Escherichia coli after Stress Exposure

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Corynebacterial recipient cells exposed to heat, organic solvents, pH shifts, or detergents show an increased fertility in subsequent intergeneric matings with Escherichia coli. This effect is independent of de novo protein biosynthesis and seems to be due to a direct inactivation of a restriction system active against foreign DNA that enters the cell by IncP-mediated conjugation.

Recent studies revealed that bacterial conjugation is a rather nonspecific process that may account for the dissemination of genetic information throughout the microbial world (see references 5, 8, and 14 for recent reviews). Moreover, it was shown that bacterial conjugation may connect the gene pools of procaryotes and eucaryotes, since bacterial plasmids could be mobilized into yeasts (9, 18) and higher plants (4). Given this ubiquity, conjugation seems to be the most important process by which horizontal gene transfer is accomplished between phylogenetically distant species in nature. Since genetically engineered DNA sequences also might be spread by conjugation, this phenomenon has gained increasing attention with regard to the release of genetically modified organisms. Despite this growing interest, very little is known about the mechanism of interspecific conjugation or about conditions affecting the frequency with which conjugal transfer occurs.

We have previously demonstrated that the mobilizable shuttle plasmid pECM1 can be transferred by conjugation from gram-negative Escherichia coli to a variety of gram-positive strains of the genera Corynebacterium, Brevibacterium, Arthrobacter, and Rhodococcus (16). We showed that the intergeneric transfer of this plasmid could be optimized by heat treating the coryneform recipient cells prior to mating. When Corynebacterium glutamicum ATCC 13032 recipient cells were heat treated for 9 min, we observed a threshold temperature of 42°C, above which a significant increase in fertility was obtained (Fig. 1a). Temperatures of 48 to 49°C gave optimal results, with $10^7$ to $10^8$ transconjugants in subsequent matings. Compared with untreated controls, therefore, an increase in fertility of 4 orders of magnitude was obtained. Temperatures above 49°C rendered a growing portion of the cells unviable and thus led to decreasing numbers of transconjugants.

In several respects, small alcohols such as ethanol display the same effects on bacterial cells as heat. Like heat, ethanol has toxic effects on cells and induces denaturation of proteins. Exposure to ethanol or heat leads to an increased ratio of saturated to unsaturated fatty acids in the cytoplasmic membrane (7, 19). Furthermore, ethanol is known to efficiently induce the heat shock response (23). We have therefore investigated whether ethanol is able to induce conjugal competence in C. glutamicum ATCC 13032. For this purpose, C. glutamicum cells grown in LB medium to logarithmic phase were mixed with twice-distilled ethanol to give final concentrations between 0 and 22% and were incubated for 9 min. Immediately after this incubation, about $4 \times 10^8$ cells were transferred to Eppendorf tubes, pelleted by centrifugation for 2 min at 14,000 rpm, and washed three times with 1 ml of LB medium each time. The cells were then used for conjugation with E. coli S17-1 containing the mobilizable shuttle plasmid pECM1 (12), as described previously (16, 17). The results of this experiment (Fig. 1b) showed that even low concentrations of ethanol are capable of increasing the number of transconjugants in subsequent matings. Optimal fertility was induced by concentrations of 16 to 20% ethanol, which led to $10^7$ to $10^8$ transconjugants. Higher concentrations were lethal to the cells. Like heat, ethanol therefore causes a high conjugal competence in C. glutamicum ATCC 13032. As was the case for heat, conjugal competence after ethanol treatment was persistent in nongrowing cultures but disappeared within 5 h in cultures allowed to grow in fresh medium (data not shown).

Protein denaturation and the release of complex stress responses are also known to occur as a consequence of exposure of cells to pH shifts or detergents (1, 10). We incubated C. glutamicum cells grown to exponential phase in the presence of different final concentrations of HCl or NaOH. After 9 min of exposure, the cells were harvested and washed twice with 1 ml of LB medium to readjust the pH to 7.5. Subsequently, these cells were used as recipients in a conjugation assay with E. coli S17-1(pECM2) as donor. As demonstrated in Fig. 1c and d, drastic pH shifts caused by acids such as HCl or bases such as NaOH also induce a significant increase in fertility. The results show that HCl is a more potent inducer of conjugal competence than NaOH, since exposure to optimal concentrations of HCl (0.07 M) gave rise to $4 \times 10^7$ transconjugants in subsequent matings compared with only $8 \times 10^6$ transconjugants after exposure to NaOH (0.08 M).

The effect of detergents on the fertility of C. glutamicum cells was studied with sodium dodecyl sulfate (SDS). A 9-min treatment of C. glutamicum cells with different final concentrations of SDS was performed. Since SDS binds firmly to the cell wall, the cells had to be washed four times with LB medium immediately after incubation to remove the detergent completely. Mating assays were then conducted with E. coli S17-1(pECM2) as the donor. Figure 1e illustrates that exposure to low concentrations of the detergent increased the fertility of C. glutamicum. Up to $3 \times 10^8$ transconjugants were achieved after treatment with 0.28 mM SDS. C. glutamicum appears to be very sensitive to SDS since concentrations exceeding 0.4 mM were lethal to a growing portion of the cells.

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Surprisingly, we achieved very high numbers of transconjugants without any preceding treatment when different restriction-deficient mutants of *C. glutamicum* ATCC 13032 (RM3 and RM4) or *C. glutamicum* AS019 (R127 and R163) were used as recipients (Table 1). These mutants display a constitutively competent phenotype, and we deduce that high fertility may be caused by impairment of the recipient's restriction functions.

To examine this hypothesis, we took advantage of bacteriophage CL31 from *Corynebacterium lilium* ATCC 15990 (24). Although the host range of CL31 was described to be restricted to *C. lilium* (24), we found that at a very low frequency it also forms clear plaques on the *C. glutamicum* wild type. A lysate of the phage from *C. lilium* (6.6 × 10^10^ PFU/ml) was used to infect the *C. glutamicum* wild type and RM3. For the infection assays, 4 × 10^7^ cells were spread onto LB agar. Subsequently, 10 μl of different dilutions of the phage lysate was spotted on the bacterial lawn, and the plates were incubated for 24 to 48 h at 30°C. Figure 2A and B demonstrates that CL31 propagates poorly on ATCC 13032, whereas it frequently infects and lyses *C. glutamicum* RM3. This indicates that the restriction system of *C. glutamicum* efficiently protects against phage infection in vivo. In Fig. 2C and D, results of infection assays performed with wild-type cells exposed to heat (panel C) or ethanol (panel D) prior to plating are shown. The assays reveal that wild-type cells become susceptible to phage infection after heat or ethanol treatment and support the hypothesis that stress may lead to an inactivation of restriction enzymes. We observed equivalent results after treatment with HCl, NaOH, or SDS (data not shown).

Alleviation of restriction may occur in the course of a complex stress response (20, 21). We added the bacteriostatic antibiotic chloramphenicol (50 μg/ml) to exponentially growing cultures of *C. glutamicum*. After addition of the antibiotic, growth rapidly declined and stopped completely within 60 min (data not shown). After this time, about 4 × 10^8^ cells were harvested, washed with LB medium to remove the antibiotic, heat treated (9 min, 48.5°C), and mated with *E. coli* S17-1(pECM2). The results shown in Table 2 implicate that de novo protein biosynthesis is dispensable for the development of conjugal competence after stress treatment. Moreover, we found a slight but significant further increase in fertility after treatment with chloramphenicol. We presume that high fertility does not evolve during a complex stress response, including de novo protein biosynthesis, but rather is due to a direct denaturation of restriction enzymes. The slight increase after chloramphenicol treatment could be explained by a delayed or diminished de novo synthesis of restriction enzymes.

### TABLE 1. Total number of transconjugants in matings of *E. coli* S17-1(pECM2) with *C. glutamicum* ATCC 13032 (wild type) and with different restriction-deficient mutants of *C. glutamicum*

<table>
<thead>
<tr>
<th><em>C. glutamicum</em> recipient strain</th>
<th>No. of transconjugants^a^</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 13032</td>
<td>3 × 10^7</td>
</tr>
<tr>
<td>RM3</td>
<td>5 × 10^6</td>
</tr>
<tr>
<td>RM4</td>
<td>5 × 10^6</td>
</tr>
<tr>
<td>R127</td>
<td>3 × 10^7</td>
</tr>
<tr>
<td>R163</td>
<td>7 × 10^6</td>
</tr>
</tbody>
</table>

^a^ For matings, 4 × 10^6^ (each) donor and recipient cells were mixed. The results are means of five independent matings. Deviations did not exceed 1 order of magnitude. Mutants RM3 and RM4 were derived from *C. glutamicum* ATCC 13032 by chemical mutagenesis with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine; R127 and R163 have been obtained by chemical mutagenesis of *C. glutamicum* AS019 (13).
while several authors already presumed that restriction could be alleviated by heat exposure (2, 6, 11, 15, 16), as far as we know this is the first report on increased conjugal fertility caused by an organic solvent, shifts to low or high pH, or detergents. We propose that restriction enzymes in *C. glutamicum* are more unstable than other cellular components or that they are localized at the cytoplasmic membrane, where they are easily accessible to effectors. Although it is generally believed that single-stranded transfer is not a target of restriction (3, 14), IncP-mediated intergeneric DNA transfer was found to be sensitive to restriction in the recipient. This agrees with another study conducted on the conjugation between *E. coli* and *Bacillus subtilis* (22). Our results question whether exchange of genetic material in bacteria might be facilitated in general by environmental stress conditions. Whether sensitivity of restriction enzymes to stress effectors is a common feature in microorganisms remains to be elucidated.

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**REFERENCES**


**TABLE 2. Influence of inhibition of protein biosynthesis prior to heat treatment on development of conjugal competence in *C. glutamicum* ATCC 13032**

<table>
<thead>
<tr>
<th>Chloramphenicol (50 μg/ml)</th>
<th>Heat treatment (9 min, 48.5°C)</th>
<th>No. of transconjugants*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>No</td>
<td>2.6 × 10⁵ ± 1.8 × 10⁵</td>
</tr>
<tr>
<td>No</td>
<td>Yes</td>
<td>4.5 × 10⁵ ± 3.0 × 10⁵</td>
</tr>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>2.0 × 10⁶ ± 1.4 × 10⁶</td>
</tr>
</tbody>
</table>

* For matings, 4 × 10⁷ cells of *E. coli* S17-1(pECM2) were mixed with an equal number of recipients. Means with deviations of five independent matings are shown. Experimental details are given in the text.

