REGULATION OF COMPETENCE DEVELOPMENT IN BACILLUS SUBTILIS

Ira C. Felkner[1] and Orville Wyss

Department of Microbiology,
The University of Texas at Austin,
Austin, Texas 78712

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Summary: The number of cells in a population of Bacillus subtilis which become competent can be either enhanced or reduced by several amino acids which includes arginine, histidine or lysine. Which effect is observed, depends upon the time of addition and the concentration. Inhibitors of protein synthesis block competence development at low concentrations during the early stages but become less effective when cells are committed to competence. These results indicate de novo protein synthesis occurs during competence development and that it is subject to regulation. Monofluoroacetate, which specifically inhibits aconitase activity also blocks competence development during these early stages. Since it prevents sporulation but has no effect on vegetative growth, some relationship between competence and sporulation through the tricarboxylic acid cycle seems to exist.

It has been reported that some of the amino acids have a positive effect on the development of competence for transfection and transformation (2, 7, 27) in Bacillus subtilis. Arginine, lysine and histidine (7, 27) are included among these. The first two and glutamate also act as regulators of aconitase and isocitric dehydrogenase in the tricarboxylic acid (TCA) cycle (9, 11). Vegetative growth does not depend on an intact TCA cycle, whereas sporulation cannot occur without it. Positive correlations between sporulation and competence were established in an earlier report (21), but fully transformable asporogenous mutants are known (20). Our experiments suggest how amino acid regulation, the TCA cycle, sporulation and competence are related.

MATERIALS AND METHODS: Bacterial strains. B. subtilis 168 ind was obtained from W R. Romig (University of California at Los Angeles). B. subtilis 569, 569 str-r and 569 ery-r were described previously (6). DNA isolation. DNA was isolated by the method of Marmur (15) or that of McDonald et. al. (16). Media. Commercial media used were Brain Heart Infusion (BHI) and Nutrient Broth. Spore stocks were main-

[1] Present address: Department of Biology, Texas Tech University, Lubbock, Texas 79409.

tained on potato extract medium (25). Minimal salts (MS) of Spizizen (22) contained 2g $(NH_4)_2SO_4$, 14g K_2HPO_4, 6g KH_2PO_4, 1g sodium citrate·$2H_2O$, 0.2g $MgSO_4 \cdot 7H_2O$ per liter of deionized, distilled water. Minimal medium (MM) was MS plus 0.5% glucose. M-1 medium was MM supplemented with 40 μg/ml tryptophan, 0.05% casein hydrolysate (CH) and 10^{-4}M $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$. M-2 medium was identical except tryptophan was reduced 8-fold and CH by 5-fold. All solid media had 1.5% Difco agar. Transformation procedures. Two procedures were used for growing competent cultures. All incubation, unless otherwise stated, was at 37 C. In regimen a, cells in BHI were shaken for 16 hr at 37 C and 0.1 ml of this suspension used to inoculate 10 ml of M-1 medium. After 4 hr incubation with vigorous shaking, cells were washed with MS and resuspended in M-2 at $OD_{\lambda 600}$ of 0.10. Incubation with shaking was continued until an $OD_{\lambda 600}$ of 0.22 was reached. For regimen b, initial growth was for 16 hr in M-2 and 0.1 ml of this suspension was added to MM, 5.0 μg/ml tryptophan and 200 μg/ml of a single amino acid. The culture was shaken for 4 hr, washed and resuspended in M-2 at $OD_{\lambda 600}$ of 0.10and like regimen a, incubated to an $OD_{\lambda 600}$ of 0.22. To insure maximum transformation, competent cells were always incubated with DNA at a concentration of 10 μg/ml (o.1 μg/ml gives maximum transformation on a saturation curve). Str-r and ery-r transformants were assessed on membrane filters (Millipore, HA type) by first incubating on BHI agar and then transferring them to BHI agar plus the selecting antibiotic.[2] After incubation for 24-48 hr transformants were scored.

RESULTS AND DISCUSSION: One cannot determine the effects of individual amino acids on competence development using regimen a for growth. However, with regimen b, only 5 μg/ml tryptophan (required by the mutant) plus the test amino acid are present. When arginine, lysine or histidine were added in stage 2 of regimen b, peak competence was reached 90 to 100 min in stage 3. The transformation frequency was always several-fold higher than it was when they were omitted (7, 27). However, the duration of the peak was considerably shorter than with regimen a and a second peak was often reached after 150 min incubation in stage 3.

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[2] Dihydrostreptomycin was 1 mg/ml and erythromycin was 1 μg/ml.

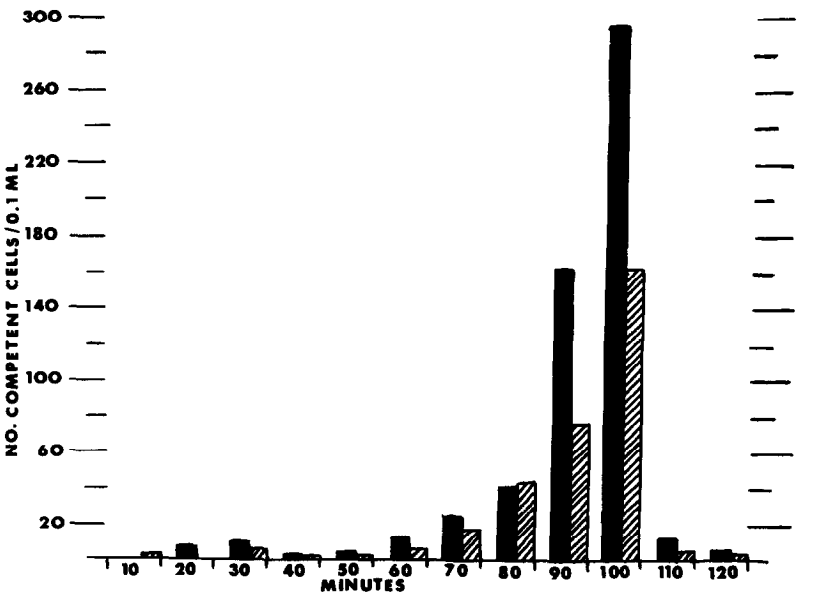

Fig. 1. Onset and loss of competence by B. subtilis strain 569 cells using lysine in the second stage of regimen b. Symbols: ■ , str-r marker; ▨ , ery-r marker. Abscissa values represent the amount of time elapsed in the final stage of regimen b before the cells were challenged with transforming DNA. Each sample plated contained about 2 x 10^6 recipient cells.

Figure 1 shows the typical pattern of competence development by regimen b for B. subtilis strain 569. Although lysine was the amino acid added during stage 2 (pre-competence), arginine and histidine give about the same pattern and maximum. Both str-r and ery-r were used for markers to insure the association of peak transformation with competence instead of time variations in marker integration (5). Differences in the number of transformants for these markers could, however, reflect integration efficiency (13).

Amino acids which can increase the level of competence when present during stage 2 of regimen b will reduce competence during stage 3 when assessment of transformation is at 90 min. The extent of reduction is dependent upon the time and con-

centration of amino acid added. Figure 2 shows transformation when 200 µg/ml of L-arginine is added at various times in stage 3 relative to the control system. Transformation is obviously more severely reduced when addition is prior to 4 min. After 30 min no significant effect is observed. This indicates that once cells are committed to competence they are no longer affected, but those in earlier stages could be blocked. This is the response expected if either allosteric inhibition (12) or enzyme repression (14, 26) were causing reduction in the number of potential transformants. Since these metabolic control mechanisms can be reversed when the responsible end product is removed through metabolism competence should develop upon continued incubation.

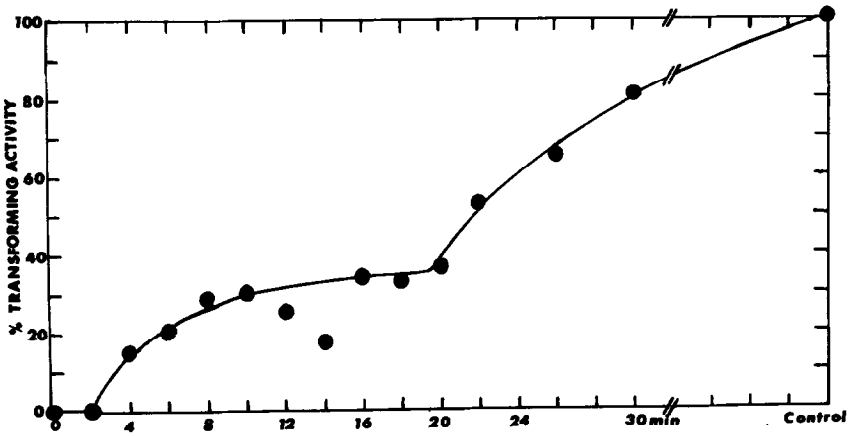

Fig. 2. Relative transforming activity of B. subtilis 168 ind grown through regimen a for competence and "pulsed" with arginine at various intervals of the final stage. All samples were assessed for transformation after 90 min growth in the final step. Str-r and ery-r frequencies were averaged over several experiments in which the control transformations were between 2 and 4×10^{-4} transformants/ml.

Cellular systems under the above regulation should be either inducible or derepressible. Although chloramphenical (CAP) has been shown to inhibit production of B. subtilis competence factor (CF_{bs}) by Akrigg et. al. (1), precise sensitivity

at the onset of competence was not established. If induction occurs by overcoming the action of a repressor (14), then low concentrations of protein inhibitors should be more effective when the process is initiated. Low concentrations (1.4 μg/ml) of CAP will inhibit inducible enzyme synthesis (18) without affecting total protein or growth, whereas 10 to 20 μg/ml will stop total protein synthesis without affecting viability of B. subtilis (17, 23). Table 1 shows that concentrations of CAP as low as 0.13 ug/ml reduced transformation about 50-fold when added at 0 min in stage 3 of regimen b. Thirty minutes later, 12.5 μg/ml caused only a 5-fold reduction. This result can be explained if de novo protein synthesis is more sensitive to CAP during initiation. Similar results were obtained with 7-Azatryptophan, also an inhibitor of protein synthesis, when varying concentrations were added during stage 3.

Table 1. The effect of chloramphenicol and 7-azatryptophan on the final level of transformation of Bacillus subtilis 168 ind.

CHLORAMPHENICOL*			7-AZATRYPTOPHAN*			
Concentration	0 min	30 min	Concentration	0 min	15 min	30 min
μg/ml	% of Control		mM	% of Control		
50	0.015	0.25	2.0	0.04	0.15	0.43
12.5	0.012	0.20	1.0	0.045	0.68	0.67
5.0	0.015	0.25	0.5	0.05	0.60	0.73
1.3	0.020	0.40	0.2	0.15	0.82	0.83
0.13	0.020	0.50	0.1	0.40	0.97	0.93
None	1.00	1.00	None	1.00	1.00	1.00

* These inhibitors of protein synthesis were added to cells up to 30 min during stage 3 of regimen b. Since all values after 30 min were around 100%, they were not included in Table 1. Control frequencies for ery-r and str-r were between 2 and 4 x 10^{-4}.

Transformation was reduced to 4% when 2mM was added at 0 min, to 15% at 15 min, and to above 40% by 30 min. When 0.2mM was added, inhibition was only significant at 0 min. This indicates that a process dependent upon new protein synthesis is essential for competence development. One possible protein could be CF_{bs} (1, 3, 8). Inhibition of protein synthesis with CAP in D. pneumoniae (24) during the interaction of cells and competence factor (CF_{dp}) blocks acquisition of competence and puromycin inhibits some de novo protein synthesis needed for induction of a molecule other than CF_{dp}. Recent experiments (Ribble, R. J. and I. C. Felkner, unpublished data) would support the idea of a critical protein molecule other than CF_{bs}.

The TCA cycle must function before sporulation can occur (9, 10, 11), and low levels of both aconitase and isocitric dehydrogenase have been linked with poor sporulation (10, 11). Since monofluoroacetate is converted to fluorocitrate and inhibits aconitase activity (4), it should inhibit processes dependent upon the TCA cycle. We have evidence (Ribble, R. J. and I. C. Felkner, unpublished data)

Table 2. Time-effect of varying Monofluoroacetate concentrations on development of competence by Bacillus subtilis strain 168 ind growing in M-2 broth.

Growth in M-2 Broth	Concentration of Monofluoroacetate (mM)			
	5	1.0	0.5	0.1
Min	% of Control Transforming Activity			
10	0.8	0.6	1.5	3.0
20	1.1	2.0	4.5	10.2
30	4.3	10.0	8.6	35.0
40	16.7	60.0	33.0	100.0
50	43.8	81.0	*100.0	93.8

* The percent transformation was in the same order of magnitude as the control population but was slightly higher.

that 1.0mM MFA prevents the appearance of proteolytic enzymes and sporulation without affecting vegetative growth. The sensitivity of competence induction to MFA during stage 3 of regimen a and b is reported in Table 2.

MFA is a more effective inhibitor of transformation prior to 30 min incubation in stage 3. Inhibition is concentration dependent within a range of 0.1mM to 5.0mM. Since preincubation in the absence of citrate allows MFA to enter the metabolic mainstream (4), citrate was withheld for the first 10 min of stage 3. Withholding citrate for up to 20 min had little effect on transformation. Completely blocking transformation with MFA does not affect vegetative growth. Since MFA is aconitase specific, an intact TCA cycle must be necessary for development of competence in B. subtilis. Experiments in progress and others in the literature show that sporulation has this same requirement. This link between these processes is even more significant since the TCA cycle is unnecessary for normal vegetative processes. Although the precise link between the TCA cycle and de novo protein is not established, the data reported here defines the period at which both are affected. This relationship is currently being investigated for both sporulation and transformation.

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