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Regulatory Mutations for Anaerobic Inducible Gene Expression in *Salmonella typhimurium*

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New regulatory loci which participate in the regulation of anaerobic inducible gene expression in *Salmonella typhimurium* were identified. We observed the regulatory network of new regulator mutations to various anaerobic inducible gene (1). Some anaerobic inducible *lac* fusions were also induced at low pH condition which was severe environment to withstand for its virulence at the place like phagolysosome. Six oxygen-regulated regulatory mutants (*oxr*) isolated by Tn10 mutagenesis were divided into two groups. Five of them were found to show negative effect on the regulation of anaerobic gene expression, while one showed positive effect on the regulation. Genetic loci of four *oxr* were identified with 54 Mud-P22 lysogens covering the whole chromosome of *S. typhimurium*, in the nearby region of map unit 87 min (*oxr*101), 63 min (*oxr*104), 97 min (*oxr*105), and 57 min (*oxr*106), respectively. Two *oxr* mutants were subjected to two-dimensional polyacrylamide electrophoretic analysis of anaerobic inducible proteins for searching the control circuitry of our *oxr* mutants.

KEY WORDS □ *Salmonella typhimurium*, *ani-lacZ* fusion, Mud-P22 lysogen oxygen-regulated regulatory mutant.

Bacteria generally respond to various types of physiological stresses including heat shock, ultraviolet irradiation, starvation, pH change, and anaerobiosis (8). *Salmonella typhimurium*, the enteric bacterium, survives successfully during the shift between aerobic and anaerobic growth, and at low pH condition to have virulence. Many gene expressions in this bacteria are controlled by the presence or absence of molecular oxygen. When grown anaerobically, many proteins involved with anaerobic respiration are induced while some proteins involved with aerobic respiration are repressed (9). Using the random lac operon fusion technique, many *ani* (anaerobic inducible) and *oxi* (oxygen inducible) genes have been characterized (2, 21).

However, the regulatory mechanism for the oxygen-regulated gene expression has been rarely characterized. The most characterized anaerobic regulatory locus is *fnr* of *Escherichia coli* (5, 11, 13). The *fnr* mutation prevents anaerobic induction of several respiratory enzymes (19, 20, 22). It has been reported in *S. typhimurium*, that several oxygen-regulated regulatory genes (*oxr*) participate in anaerobic transcriptional control. These include *oxr*A, homologous loci to *fnr* of *E. coli*, *oxr*B, *oxr*C (*pgi*), *oxr*D, *oxr*E (*ear*A), *oxr*F, and *oxr*G (3, 12, 23).

In the previous study, we have constructed and identified 13 *ani-lacZ* operon fusions added to already known 16 *ani-lacZ* fusions in *S. typhimurium* (1). To search for the regulatory loci controlling anaerobic gene expression, we utilized six *ani-lacZ* operon fusions that showed different regulatory characteristics. Recently, there have been the reports about that *S. typhimurium* has an adaptative reponse to extreme acidic pH condition, which provides an explanation of its survival from this harsh *in vivo* environment like in macrophage phagolysosome (6). So, we checked if our *ani-lacZ* fusions are induced by low pH, looking for commonly responsible genes at both conditions. This report introduces new *oxr* mutations and several acid-inducible *ani-lacZ* fusions, and provides regulatory network about anaerobiosis and pH induction. Finally, we analysed anaerobic inducible proteins of our *oxr* mutant by two dimensional polyacrylamide gel electrophoresis (PAGE) and determined which are controlled by our *oxr*s.

MATERIALS AND METHODS

Strains and bacteriophages

The bacterial strains and bacteriophages used in this study are listed in Table 1. Phage P22HT 105/1-int, and its derivative H5, were used for general transduction and nonlysogen test, respetively.

Media and cultural conditions

Cultural conditions including LB medium, minimal E medium, MacConkey medium, Green plates for phage sensitivity were used as descrived early (1, 2). NCE-lactose minimal medium for spontaneous mutation, MOPS (Morpholine propane sulforic acid) for labelling with S^{35}-methionine were used. Bochner medium was used for mapping the Tn10 insertions as described by Nicholas *et al.* (18). Decarboxylase base moeller media for identifing amino acid decarboxylase gene were used as described by Auger *et al.* (6). Antibiotics were used at the concentration of 30 μg/ml for ampicilin, 20 μg/ml for kanamycin, 20 μg/ml (complex medium), 10 μg/ml (minimal medium) for tetracycline.

For anaerobic condition, 1 ml of paraffin oil was overlayed to liquid culture and Gaspack Anaerobic system (BBL Microbiology system) was used for plate.

Genetic manipulation

General transduction with P22HT105/1-int was performed as described previously (1, 17). After transduction, nonlysogenic strains were identified by cross-streaking against phage H5 on the green indicator plate.

Isolation of regulatory mutants

Two methods were used. The first method used to isolate regulatory mutants involved screening a random pool of Tn10 insertions into regulatory loci (17). P22HT105/1-int propagated on the Tn10 insertion pool randomly inserted into *S. typhimurium* chromosome was plated with *ani-lac*Z recipients onto MacConkey Tc plate. Duplicated plates were incubated aerobically (looking for constitutive expression of β-galactosidase) and anaerobically (looking for a lack of anaerobic induction). The second method involved plating 10^7 cells of *lac*Z fusion strains onto NCE-lactose medium with incubation under repressive condition (aerobically for *ani-lac*Z strains). This method was used to search for spontaneous regulatory mutations which express constitutively *lac*Z fusion, resulting in β-galactosidase levels suitable to support growth on lactose as a sole carbon source (3).

Effects of isolated regulatory mutants to various *ani-lac*Z fusion strains were analysed by β-galactosidase activity test (17).

Linkage mapping of oxygen-regulated genes

For linkage mapping of our *ani, oxi-lac*Z fusions, strains having minitransposon Tn10Δ16Δ17 randomly inserted on the chromosome of *S. typhimurium* (4). Phage P22 HT propagated on our Mu dJ(Km, *lac*) operon fusions were plated with overnight culture of the Tn10Δ16Δ17 known insertion strain onto LBKm. Selected Km^r colony was replicated to LBTc, then cotransduction frequency was calculated by using Kemper's formula (1).

Table 1. *Bacterial strains and Bacteriophages*

Strain or Phage	Genotype	Source
Strains		
S. typhimurium LT-2	wild type	SGSC[a]
AK3131	*zad*-3131 (linked 24% to *leuBCD* by P22)	SGSC
AK3150	*zah*-3150 (linked 82% to *proAB* by P22)	SGSC
Mud-P22 Mapping Strains		SGSC
YK109	*ani*2001::Mu dJ	Our Lab.
YK126	*ani*2005::Mu dJ	〃
YK127	*ani*2006::Mu dJ	〃
YK130	*ani*2009::Mu dJ	〃
YK131	*ani*2012::Mu dJ	〃
YK132	*ani*2013::Mu dJ	〃
YK211	*oxi*3011::Mu dJ	〃
Bacteriophages		
P22	HT 105/1-int	J.W. Foster
H5	Derivative of P22	〃

[a]SGSC: Salmonella Genetic Stock Center at Univ. of Calgary, Canada.

Rapid mapping of *oxr* genes with Mud-P22 prophages

Youderian and colleagues *et al.* (24) constructed a set of map, locked in Mud-P22 prophages (MudP: clockwise, MudQ: counterclockwise) along the *S. typhimurium* chromosome. Each of 54 prophages was able to package about 3 minutes of chromosomal DNA adjacent to the site of prophage insertion. For preparing each lysate of each Mud-P22 lysogens, the procedure of Nicholas *et al.* (18) was used.

oxr mutants carrying the Tn10 to be mapped was grown to 4×10^8 cells/ml in LB medium containing tetracyclin. The cells were washed twice in phosphate-buffered saline and diluted 10-fold, and 0.1 ml cells were plated on fresh Bochner plates. Each 5 μl spot of 15-fold diluted all the Mud-P22 lysates was applied to prespread Bochner plates. The plates were incubated at 37℃ for 24 hr. The Mud-P22 insertion close to the site of Tn10 insertion gave a confluent spots of Tet^r transductants.

Two-dimensional polyacrylamide gel electrophoresis

The procedure for two-dimensional electrophoresis of cellular proteins was as descrived by Spector *et al.* (3, 21). Cells were grown in MOPS medium with vigorous aeration to 2×10^8 cells/ml. Then cells were labelled with 50 μCi/ml of $H_2{}^{35}SO_4$

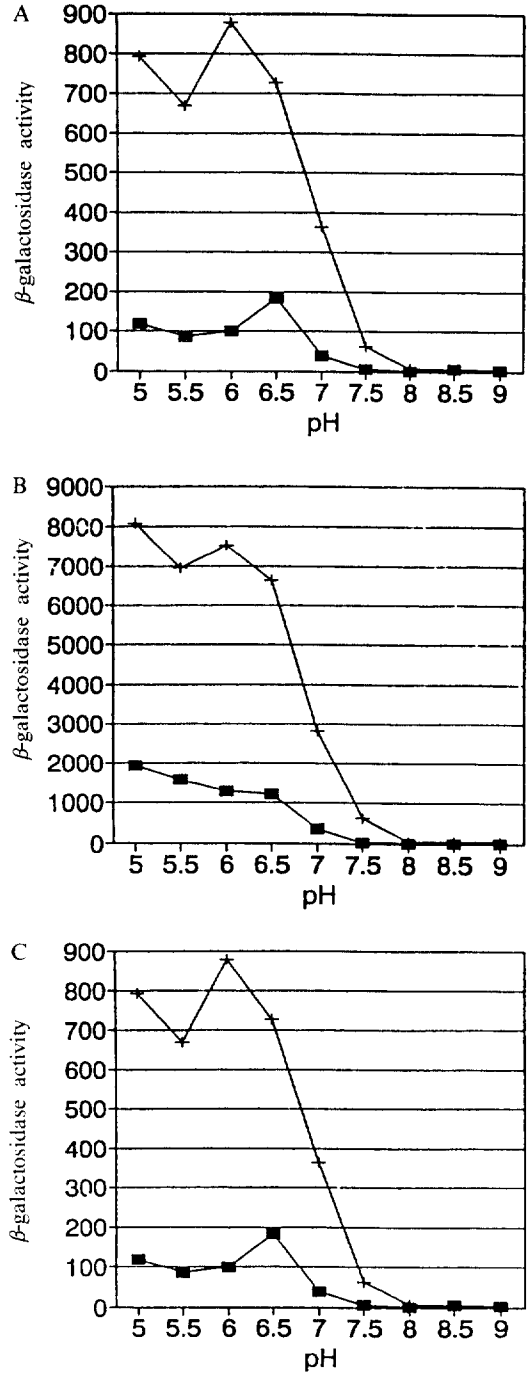

Fig. 1. *Transcriptional control of ani-lacZ fusion strains by external pH.*
(Cells were grown to equivalent densities under aerobic and anaerobic conditions in LB medium containing MES or MOPS buffers) +: aerobic, ■: anaerobic, A: YK126, B: YK 131, C: YK132

Table 2. *Decarboxylase test of pH regulated operon fusions*

Strain	Genotype	Decarboxylase base moeller		
		1% lysine	1% argine	1% ornithine
LT-2	wild type	P	V	P
YK112	*ani*2004::Mu dJ	P	V	P
YK124	*ani*2002::Mu dJ	P	V	P
YK125	*ani*2003::Mu dJ	P	V	P
YK126	*ani*2005::Mu dJ	P	V	P
YK131	*ani*2012::Mu dJ	Y	V	P
YK132	*ani*2013::Mu dJ	P	V	P

P: purple, V: violet, Y: yellow

and splited: 2 m*l* of cells were left under continuous aerobic culture and the other 2 m*l* were transfered to culture tube and overlayed with paraffin oil, then grown for one generation time.

About 1.5 m*l* of labeled cells was pelleted and suspended in 13 μ*l* of sodium dodecyl sulfate (SDS) lysing solution. They were boiled for 5 minutes and run in a pH 5 to 7 isoelectric focusing system followed by 11.5% SDS-PAGE. Comparisons were made between aerobic and anaerobic samples with equivalent protein (5~15 μg) at the base on the coordinates of standard two-dimensional profiles of *S. typhimurium* made by Spector *et al.* (21).

RESULTS AND DISCUSSION

Effect of the external acidic pH to *ani-lacZ* fusion strains

External pH has been implicated as a signal in a growing number of genetic responses including the pathogenesis in some enteric bacteria. Sometimes they encounter both anaerobic and low pH conditions as well as other harsh stresses to overcome for their virulence (15). To observe the effect of pH change on the anaerobic gene expression, all *ani-lacZ* fusion strains we've got were challenged to a wide range of pH (Fig. 1). Some of them, YK126, YK131, and YK132, also showed high induction fold of β-galactosidase activity at the external acidic pH condition (Fig. 1). Because it has been known that several amino acid decarboxylase genes were induced under anaerobic conditions at low pH (6, 7), the above fusion strains were subjected to decarboxylase indicator media in order to verify amino acid decarboxylase gene deficiency (Table 2). Only YK 131 showed lysine decarboxylase deficiency, and the other strains were not related to decarboxylase genes. The result obtained so far indicated that there is another gene group or modulon for induction at both anaerobic and low pH con-

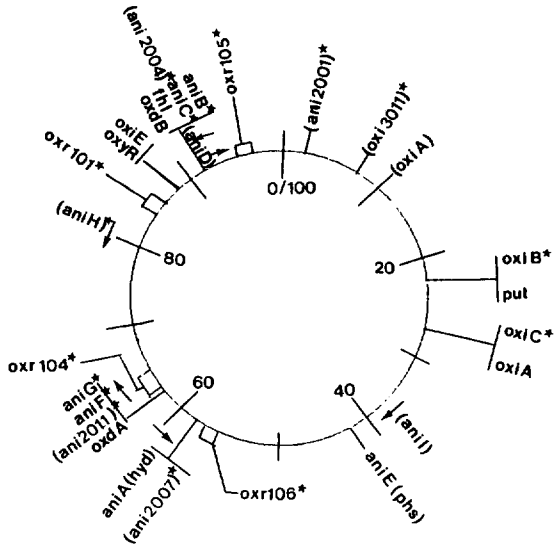

Fig. 2. *Linkage map of oxygen-regulated loci and mapping region of oxr genes in S. typhimurium.* The relationship of loci marked with an asterisk to adjacent marks is not known. Map locations of loci in parentheses are approximate.

ditions in *S. typhimurium* different to the group of amino acid decarboxylase, and that some signal sensing system of bacterial cell could simultaneously respond to two or more stresses.

Linkage mapping of ani, oxi genes by cotransduction frequency

Two oxygen-regulated genes not identified its location on the chromosome were mapped by cotransduction with strains having Tn10Δ16Δ17 insertion on the chromosome (4). YK109 (ani 2001) was linked 16% to Zad-3131 (AK3131), and YK211 (*oxi* 3011) was 3% linked to Zah-3150 (AK 3150). The loci of these genes were calculated by cotransduction frequency with Kemper's formula (1), 2 min and 7 min, respectively (Fig. 2). These loci were identified as new oxygen-regulated gene.

Isolation of oxr mutation

In order to isolate *oxr* mutant from *ani-lac*Z fusions, YK109, YK126, YK127, YK130, YK131 and YK132, spontaneous mutation and Tn10 mutagenesis were used as described in Materials and Methods. Two *oxr* mutants were isolated by spontaneous mutation from YK127 (Table 3). To determine whether these mutation were linked to *ani*::Mu dJ (Km, *lac*) (possibly representing promoter or operator regions) the *ani*::MudJ from each regulatory mutant was transduced into *S. typhimurium* LT-2, and Km^r transductants were subsequently scored for oxygen regulation. These mutants were identified to contain mutations linked to *ani*::Mu dJ (presumably promoter or operator mutations). By using Tn10 mutagenesis six *oxr* mutants were isoloated; *oxr*101 and 102 from YK109, *oxr*103 and 104 from YK127, *oxr*105 from YK130, and *oxr*106 from YK132 (Table 3). These strains containing Tn10 insertions located near *oxr* were examined whether Tn10 insertions were linked to each of *ani* genes or not. P22 propagated on Tn10 insertion strain was transduced to parent *ani-lac*Z fusions. All the Tn 10 insertion for *oxr* was identified 100% linked to Tc^r and *oxr*$^-$ phenotype (Its location on the chromosome was trans-regulator loci). Former five mutants negatively regulated *ani-lac*Z fusion and *oxr* 106 showed positive regulation (a lack of *ani-lac*Z induction at the anaerobic condition in Table 3). It has been reported that the regulation of anaerobic stimulon was controlled by *oxr*A, *oxr*B, *oxr*C, tppR, *oxr*G, *ear*A and *oxr*F, and one regulator simultaneously controled many oxygen-regulated genes(e.g. *oxr*A-*ani*A, *ani*C) or one gene

Table 3. *Regulatory mutations affecting the expression of ani-lacZ fusion*

Strain	Genotype	β-galactosidase activity	
		Aerobic	Anaerobic
YK109	*ani*2001::Mu dJ	35	857
YK1001	*ani*2001::Mu dJ *oxr*101::Tn10	785	831
YK1009	*ani*2001::Mu dJ *oxr*102::Tn10	629	823
YK127	*ani*2006::Mu dJ	7	552
YK1011	*ani*2006::Mu dJ *oxr*103::Tn10	406	542
YK1014	*ani*2006::Mu dJ *oxr*104::Tn10	512	580
YK1017	*ani*2006::Mu dJ *ani*2006^{po}111	694	684
YK1019	*ani*2006::Mu dJ *ani*2006^{po}120	601	733
YK130	*ani*2011::Mu dJ	48	984
YK1025	*ani*2011::Mu dJ *oxr*105::Tn10	835	952
YK132	*ani*2013::Mu dJ	31	759
YK1037	*ani*2013::Mu dJ *oxr*106::Tn10	6	8

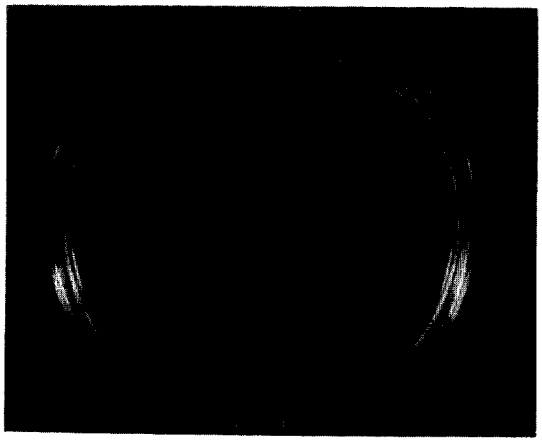

Fig. 3. *Rapid Mapping of oxr101::Tn10 mutation by Mud-P22 lysates. The lysates were diluted 15-fold and 5-μl volumes were spotted as described in Materials and Methods. Arrowhead indicates confluent spots where Tn10 was exchanged by one of Mud P22 prophages.*

was controlled by serveral regulator (e.g. tppB-tppR, *oxr*C) (3, 12, 23). Therefore, our *oxr* mutations introduced to other *ani-lacZ* fusions different from parent *ani-lacZ* strain, but our *oxr* mutation did not affect to other *ani-lacZ* strains (data not shown).

And the more we examined the effect of our *oxr* mutants at the external acidic pH condition, any *oxr* strain was not coregulated with low pH. Although there was coregulator with both anaerobic and low pH condition like *ear*A (3), our *oxr* participated only in oxygen regulation. The regulator mutation *oxr*106, for YK132 which induced at both anaerobic and low pH, did not show any difference at the acidic pH condition. These results showed that there was more *oxr* gene than known *oxr*s and regulatory pathway would be more complexed, not formulated as described previously (2, 3).

Rapid mapping of Tn10 insertions into *oxr*

To map the regulatory loci, we used recently constructed the P22/Mu hybrid pahge, Mud-P22 prophages, packaging the chromosome of *S. typhimurium* on both transcriptional direction. If the homologous recombination between Tn10 insertion near *oxr* region and one of 54 Mud-P22 lysates occurred, Tn10 could be exchanged by Mud-P22. Then *oxr* Tn10 strain is to Tet^r transductant and represents confluent spot on Bochner's media, while the remaining spots appeared similar to the background of cells that did not receive any phage (Fig. 3). By using this method, newly isolated *oxr* mutation mapped in the region of map unit 86 min for *oxr*101, 63 min for *oxr*104, 97 min for *oxr*105 and 57 min for *oxr* 106, respectively (Fig. 2). These loci were revealed to new loci of *oxr* mutation compared to already

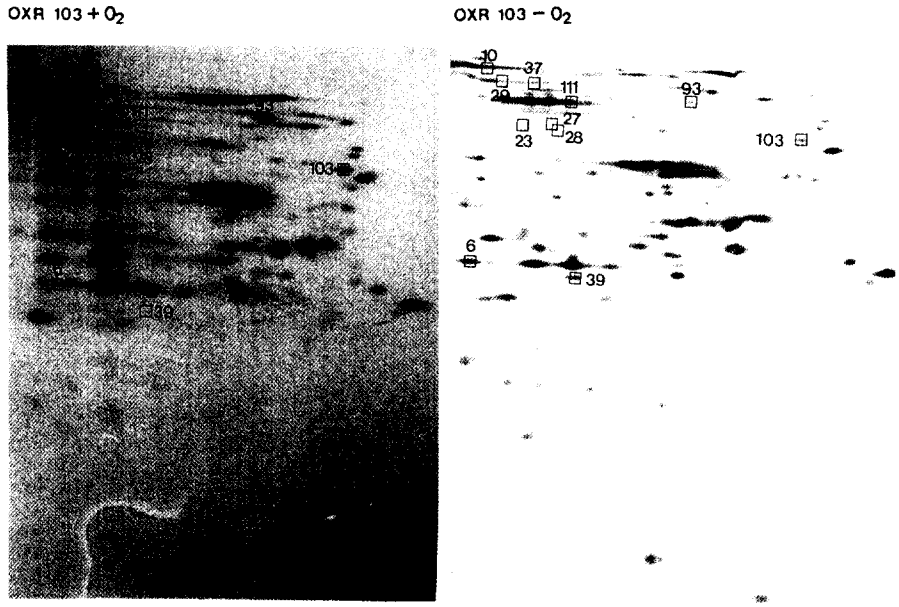

Fig. 4. *Two-dimensional patterns of anaerobiosis-inducible proteins from oxr103 mutants. SDS-boiled lysates were prepared as described and run in a two-dimensional system including a pH5 to 7 (right to left) isoelectric focusing gel followed by 11.5% SDS-PAGE. Acidic proteins are situated to the right of each gel.*

Table 4. *Effects of various regulatory mutations on the two-dimensional profile of anaerobiosis-inducible proteins*

Protein no. and designation[a]	Induction[b]						
	*oxr*103	*oxr*A	*oxr*B	*oxr*C	*ear*A	*oxr*F	*oxr*G
6.ANI-1	C	N	N	C	N	N	N
10.SIN-27[c]	C	N	N	N	N	N	N
23.ANI-6	N	−	−	−	N	N	N
27.ANI-8	N	N	N	N	N	N	N
28.ANI-9	N	N	N	N	N	N	N
29.ANI-10	C	−	N	+/−	N	N	N
37.ANI-11	C	N	N	C	N	N	N
39.ANI-12	N	N	N	C	N	N	N
93.ANI-21	+/−	N	N	N	N	N	N
103.ANI-24	+/−	−	−	N	N	N	N
111.ANI-27	N	−	−	−	−	−	−

[a]: From reference 21
[b]: N, Normal induction; −, no induction; C, constituve; +/−, induction than normal.
[c]: SIN, starvation-inducible protein.

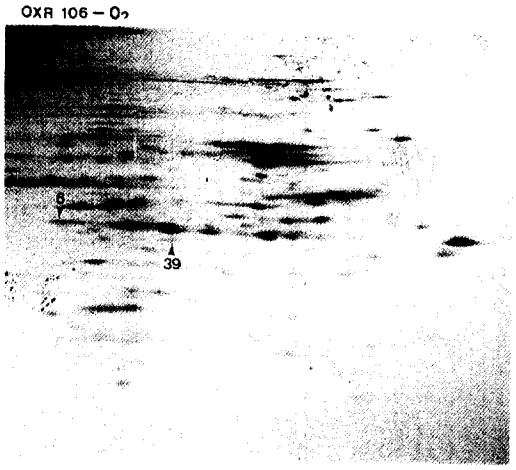

Fig. 5. *Two-dimensional patterns of ANI proteins not induced at the anaerobic condition from YK 1037(ani2013::Mud J oxr106::Tn10) mutants. Arrowheads designate repressed proteins.*

identified loci of other *oxr* mutations.

Because there was *pro*U locus, an potential osmoregulatory gene, in the mapping region of the positive regulator *oxr*106, *pro*U::Tn10 lysate, was transduced to YK132 (parent strain of *oxr*106) and examined its effect to *ani-lac*Z expression. But there was not any *pro*U effect to anaerobic expression of *ani-lac*Z. It was shown that these two genes were close each other on the chromosome of *S. typhimurium*.

Two-dimensional electrophoretic analysis of anaerobiosis inducible proteins

The purpose of this procedure was to map the controlling circuitry involved with anaerobic gene expression. YK1011 (*oxr*103::Tn10), one of the negative regulatory mutant, was subjected to two-dimensional electrophoretic analysis of cellular proteins (Fig. 4). The coordinates were used from standard two-dimensional profile of anaerobiosis-inducible proteins as described by Spector *et al.* (21).

Analysis of the *oxr* 103 gels showed different patterns to those of known *oxr* mutation (Table 4). The comparison of proteins between *oxr* 103 and other known *oxr* mutations were made according to the protein profiles by Spector *et al.* (21). Eleven proteins were regulated by *oxr* 103 and eight of them showed the different regulatory pattern compared to those of known *oxr* mutation. It implied that this mutation had two prominent controlling boundary about oxygen regulation. At the other hand, a positive regulator, *oxr*106, repressed several proteins normally induced in anaerobic culture (Fig. 5). ANI-27, ANI-1, ANI-12, and ANI-7 were normaly induced by various *oxr* mutation, but analysis of *oxr*106 gel showed repressive pattern of these proteins. Although we did not get the aerobic coordinates yet, *oxr*106 was thought to a potential regulator ever before.

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초 록: *Samonella typhimurium*에서 무산소 유도 조절유전자의 분리 및 발현

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여러개의 *ani-lac*Z 융합 균주들을 pH, 산소, 무산소 변화에 대해 조사하였으며 무산소조건에서 발현이 유도되는 gene의 조절에 관여하는 몇개의 조절유전자를 *Salmonella typhimurium*에서 새롭게 동정, 분석하였다. 또한, 기존의 무산소 유도 *ani-lac*Z 융합균주 중 산성 pH에 의해서도 유도되는 3개의 균주를 확인하였다.이들은 YK126, YK131, YK132로써, 무산소 조건뿐 아니라 산성 pH조건에서도 높은 β-galactosidase 유도비를 나타내었다. Tn10을 이용해서 분리한 6개의 *oxr* 돌연변이 균주는 두개의 군으로 나누어 진다. 그들 중 5개는 음성적으로 조절하였고 나머지 1개는 양성적으로 조절하였다. 네개의 *oxr*의 유전자좌는 87 min(*oxr*101), 63 min(*oxr*104), 97 min (*oxr*105), 57 min(*oxr*106)으로 54개의 Mud-P22 lysogen에 의해 확인되었다. 2차원 폴리아크릴아마이드 전기영동(2-D PAGE)을 이용하여 무산소 유도 단백질에 대한 *oxr* 돌연변이의 조절양상을 확인하였다.