Supplemental Materials

Materials and Methods

Strategy for genome reduction

Single-deletion strains as to each candidate region were prepared by the markerless deletion method (Supplemental Table 1). The method consists of two recombination events stimulated by λ Red recombinase (Supplemental Figure 1). For the first recombination a target region was replaced with a marker cassette (SC fragment) to make a SC deletion strain, and for the second one, the marker cassette was eliminated to make a PO deletion strain (Supplemental Figure 1). The SC fragment included the chloramphenicol resistant gene (*cat*) and a negative selection marker (*Bacillus subtilis sacB*). Since *sacB* makes *E. coli* sensitive to sucrose, a markerless deletion strain (a PO deletion strain) was successfully selected using its sucrose-resistant phenotype without the negative selection marker, *sacB* (Supplemental Figure 1). This method takes one week per one markerless deletion. The obtained deletion strains and the results of growth tests are summarized in Supplemental Table 1. Initial regions selected for deletion numbered 103. Among them, ten initial designs were shortened (altered designs are indicated by "*" in Supplemental Table 1) to obtain deletion strains. Finally, 102 deletion mutants were actually obtained, which included 84 showing normal growth in M9 minimal medium like the parental strain W3110red. Deletions located within 50 kbp were integrated in a single genome through multiple rounds of markerless deletions of targeting regions (Supplemental Table 2). All single-deletion and deletion-unit strains were strictly assessed for growth in M9 minimal medium. Regions which did not affect growth were

used to construct multiple deletion strains. The growth test conditions were as follows: Fifty-μl seed culture was inoculated into a test tube containing 5-ml M9 minimal medium. The cultivation conditions were 37℃ and 250 rpm. Two hundred-μl culture was diluted with 1.8-ml 0.1 N HCl, and then the optical density at 660 nm (OD_{660}) was measured at 6, 10, 13, 16, 19 and 32 hours to evaluate growth.

Construction of an *E. coli* cell with a reduced genome

Three new single deletions or deletion-units were transduced using the P1 phage from the original deletion mutants to the recipient strains in order to accumulate new deletions. A single deletion strain or a deletion-unit strain with the SC marker cassette was integrated into a recipient strain. Mutants with an additional deletion were selected as to their chloramphenicol-resistance, and the marker cassette was eliminated by sucrose-resistance. A family tree of deletion strains is shown in Supplemental Figure 2. Strains 22stepA, B, and C hardly accepted an additional deletion. The main reason of incompetence for further deletion was Δ043. So the Δ043 region was reverted to the wild type. Finally, 53 deletions were combined in one strain via 28 cycles of deletion-transfer (Figure 1). The resultant strain was designated as MGF-01. The composition of reduced genome *E. coli* is shown in Supplementary Table 3.

Supplemental Figure Legends

Supplemental Figure 1. Schematic illustration of the two-step markerless deletion method.

In the first recombination, a target region is replaced and deleted by the SC fragment. Deletion mutants were selected as chloramphenicol-resistant transformants and further checked as to sucrose-sensitive phenotype. In the second recombination, the inserted SC fragment is replaced and deleted by the PO fragment. Markerless deletion mutants were selected as sucrose-resistant transformants and further checked as to chloramphenicol-sensitive (Cm^S) phenotype.

Supplemental Figure 2. Lineage of strain MGF-01. A deletion unit (unit A to T in Table 2) or a single deletion (Δ001 to Δ109 in Table 1) was integrated into a proximate strain linked by a line. The names of the resulting strains are indicated below the names of the newly integrated deletion units or single deletions. Strains directly linked to MGF-01 are indicated in bold letters.

Supplemental Table 1. Single deletion strains.

deletion name[a]	left	right	length	clone[b]	growth[c]	integrated in MGF-01[d]
Δ101	15035	20289	5255		–	
Δ101*	19460	20289	830			
Δ102	71935	77094	5160			
Δ103	167065	172979	5915			○
Δ001	244301	253746	9446			○
Δ002	262300	387867	125568			○
Δ007	389475	404039	14565			○
Δ008	471822	490480	18659	–		
Δ009	518366	533047	14682			○
Δ010	535841	550551	14711			○
Δ011	564277	608454	44178			○
Δ109	616865	623504	6640		–	
Δ012	675440	689710	14271			○
Δ013	729157	739929	10773			○
Δ104	793553	798837	5285			
Δ014	832889	848426	15538			○
Δ015	867975	883810	15836			○
Δ016	891335	909716	18382			○
Δ017	993702	1005264	11563		–	
Δ017*	993702	997288	3587			
Δ018	1049225	1097357	48133			○
Δ089	1127734	1142523	14790		–	
Δ090	1180190	1191994	11805			
Δ019	1198444	1225484	27041			○
Δ020	1228177	1244657	16481		–	
Δ020*	1228177	1231892	3716			
Δ091	1296040	1308865	12826			
Δ021	1353109	1367223	14115		–	
Δ022	1371903	1437413	65511			
Δ023	1444765	1459211	14447			
Δ024	1463839	1483912	20074			
Δ025	1493425	1501185	7761			
Δ026	1503975	1537585	33611			
Δ027	1558830	1572320	13491			
Δ028	1574125	1607784	33660	–		
Δ029	1624348	1640177	15830	–		○
Δ029*	1630051	1640177	10127			
Δ030	1641771	1649361	7591			○
Δ031	1719063	1729955	10893		–	
Δ031*	1722176	1729955	7780			
Δ105	1797915	1800238	2324			
Δ032	1818151	1823955	5805			
Δ033	1825151	1843865	18715		–	
Δ034	1867443	1888554	21112			
Δ092	1903746	1907820	4075			
Δ035	1916543	1927807	11265		–	
Δ035*	1916543	1926625	10083		–	
Δ036	1963663	1992214	28552			○
Δ037	1995801	2027348	31548			○
Δ038	2032588	2043515	10928			○
Δ039	2068288	2081065	12778			○
Δ040	2103532	2115203	11672			○
Δ041	2145403	2186994	41592			○
Δ042	2199808	2228989	29182			○
Δ093	2240080	2244979	4900			
Δ088	2255034	2262630	7597			○
Δ043	2269579	2285727	16149			
Δ106	2303383	2305962	2580			
ΔK	2324713	2339072	14360			
Δ044	2366102	2378285	12184		–	
Δ045	2385401	2394610	9210			○
Δ046	2422504	2433237	10734			○
Δ047	2471991	2481621	9631			○
Δ094	2504214	2513696	9483			○
Δ048	2550201	2558664	8464		–	
Δ107	2552267	2556111	3845		–	
Δ095	2671911	2682709	10799			
Δ050	2754815	2788618	33804			○
Δ051	2794312	2800028	5717			○
Δ096	2803446	2809285	5840			○
Δ097	2824452	2829398	4947			○
Δ052	2875237	2886183	10947			
Δ053	2890870	2905236	14367			
Δ100	2909708	2910007	300			○
Δ054	2984498	3032266	47769			○
Δ098	3076224	3078055	1832			○
Δ055	3109246	3133479	24234			○
Δ056	3164770	3172081	7312			○
Δ057	3174759	3182375	7617	–		
Δ058	3183306	3193120	9815	–		
Δ059	3220176	3258311	38136			
Δ060	3271481	3304307	32827	–		
Δ060*	3271481	3298004	26524			
Δ061	3336821	3344521	7701	–		
Δ062	3361081	3370891	9811			○
Δ063	3391906	3405315	13410	–		
Δ064	3411502	3421221	9720			○
Δ080	3486425	3496907	10483			○
Δ079	3536154	3549688	13535			○
Δ078	3556476	3576240	19765			○
Δ077	3697303	3709528	12226			○
Δ076	3730161	3746404	16244	–		
Δ075	3762907	3772818	9912			○
Δ074	3774085	3785258	11174			○
Δ073	3832314	3847588	15275		–	
Δ073*	3832370	3843413	11044		–	
Δ072	3868669	3896674	28006			
Δ071	3900889	3915001	14113	–		
Δ070	3932708	3991889	59182		–	
Δ070*	3932708	3970896	38189		–	
Δ069	4003003	4021348	18346			○
Δ068	4040696	4062630	21935		–	
Δ068*	4053129	4062630	9502			
Δ066	4066911	4105898	38988	–		
Δ065	4170561	4186910	16350			○
Δ081	4239494	4255870	16377			○
Δ082	4303001	4316734	13734			○
Δ083	4318541	4334945	16405			○
Δ084	4414759	4428377	13619			○
Δ108	4461354	4465751	4398			
Δ099	4467771	4472020	4250			
Δ085	4501438	4514770	13333			○
Δ086	4523112	4604872	81761			○

[a]Names of deletion units. Deletions that defined the end positions are indicated by large capitals at the ends of their names.

[b]Deletion clones that could not be obtained are indicated by minus signs (-).

[c]Deletion clones with worse growth in M9 minimal medium are indicated by minus signs (-).

[d]Deletions integrated to MGF-01 are indicated by circles.

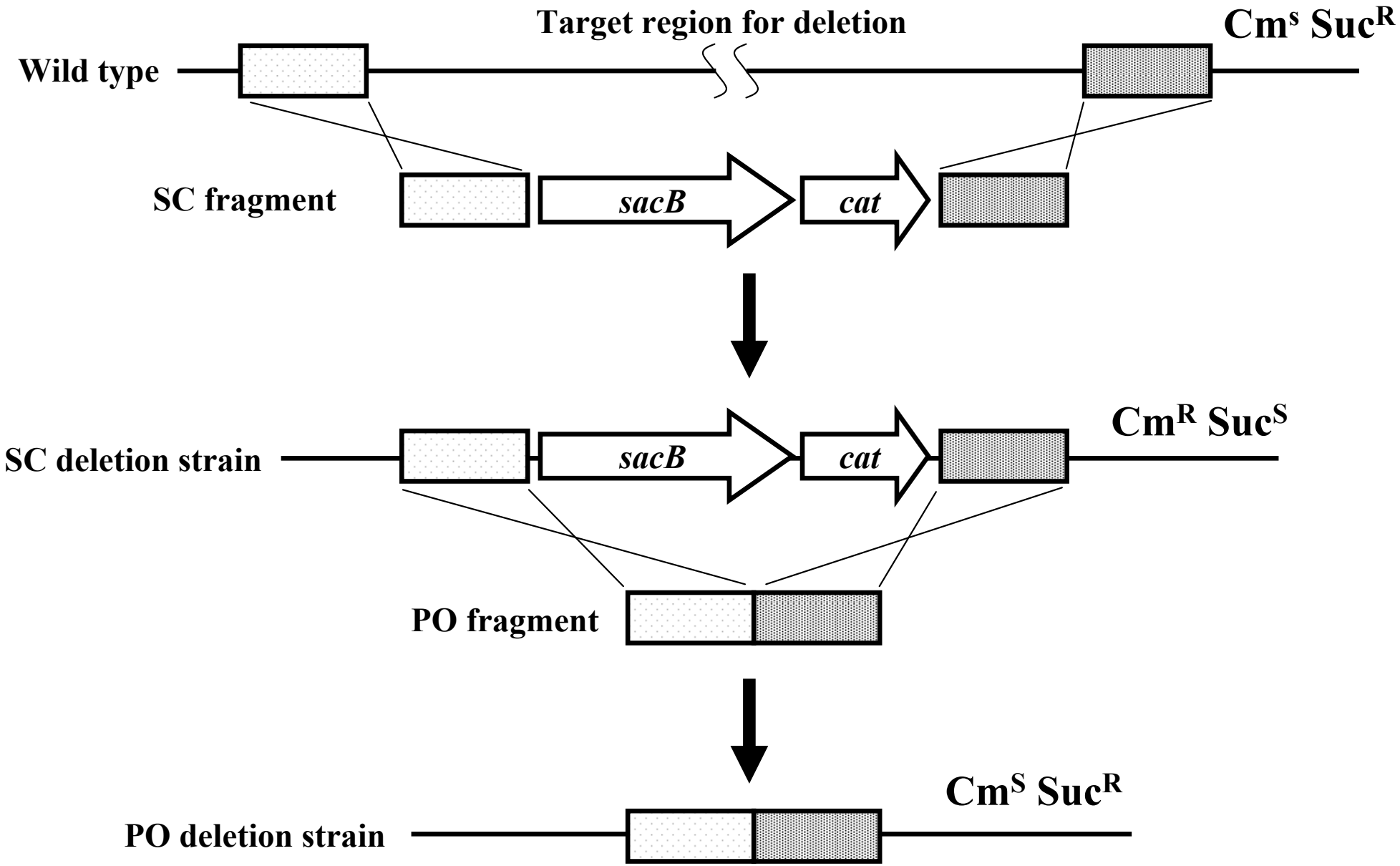
Supplemental Table 2. Components of deletion units.

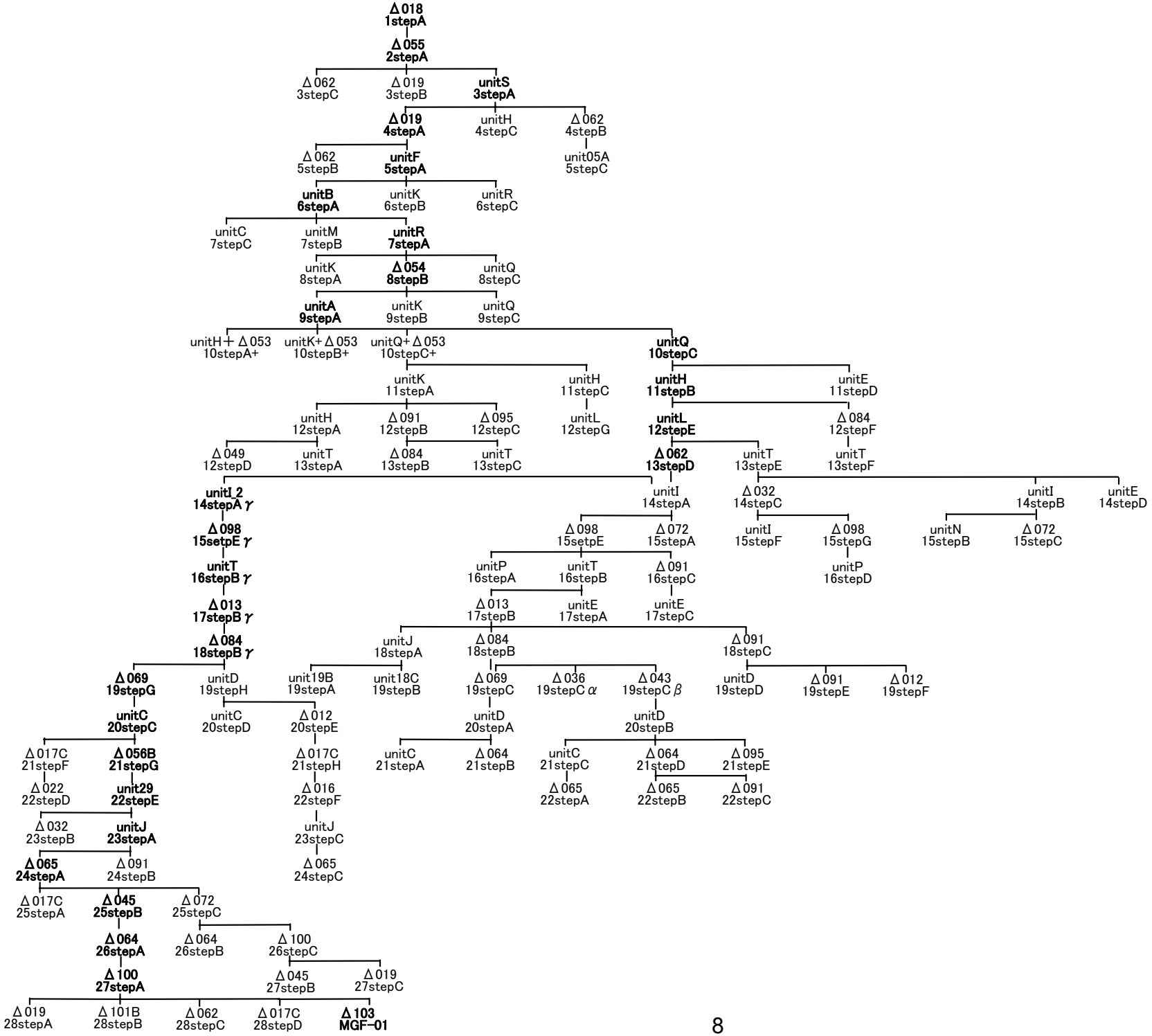
	plan	refined and fixed design[a]	integrated in MGF-01[b]
unit A	Δ002_Δ007_Δ001	same as plan	○
unit B	Δ009_Δ010_Δ011	same as plan	○
unit C	Δ014_Δ015_Δ013_Δ012_Δ016	Δ014_Δ015_Δ016	○
unit D	Δ022_Δ024_Δ021	Δ022_Δ024	
unit E	Δ025_Δ026_Δ027	Δ025_Δ026	
unit F	Δ029_Δ030	same as plan	○
unit G	Δ032_Δ033_Δ034_Δ092		
unit H	Δ036_Δ037_Δ038_Δ039_Δ040	same as plan	○
unit I	Δ041_Δ042_Δ043_Δ088_Δ093	Δ041_Δ042_Δ043_Δ088	
unit I-2		Δ041_Δ042_Δ088	○
unit J	Δ K_Δ044_Δ045_Δ046_Δ047_Δ094	Δ046_Δ047_Δ094	○
unit K	Δ048_Δ049		
unit L	Δ050_Δ051_Δ096_Δ097	same as plan	○
unit M	Δ052_Δ053_Δ054		
unit N	Δ059_Δ060B_Δ056B		
unit O	Δ064_Δ065		
unit P	Δ070_Δ069_Δ072_Δ073		
unit Q	Δ074_Δ075_Δ077	same as plan	○
unit R	Δ078_Δ079_Δ080	same as plan	○
unit S	Δ082_Δ083_Δ081	same as plan	○
unit T	Δ085 Δ086	same as plan	○

[a]Deletion unit clones which did not show good growth in M9 minimal medium showed changes their combinations of deletions from the plan. Clones that were constructed according to the plan are indicated in bold type.
[b]Deletions integrated to MGF-01 are indicated by circles.

Supplemental Table 3. Composition of reduced genome strains.

Gene product type	W3110	MGF-01	Δ16	MS43
Enzyme	1094	970	888	1014
Enzyme, predicted	390	300	266	334
Transporter	343	257	246	315
Transporter, predicted	254	172	169	219
Regulator	243	191	167	218
Regulator, predicted	164	110	110	137
Membrane	43	41	37	42
Membrane, predicted	210	166	153	181
Factor	150	131	121	138
Factor, predicted	60	50	54	56
Structural component	89	71	81	62
Structural component, predicted	37	35	32	33
Carrier	77	70	50	74
Carrier, predicted	42	33	32	40
Lipoprotein	46	39	36	42
Cell process	56	50	53	49
Leader peptide	11	11	11	11
Pseudogenes in common	74	30	44	44
Phage/IS in common	310	81	117	19
Phage/IS in common, predicted	10	6	5	5
Partial information	146	108	113	130
Unknown function	471	346	339	391
No classification	76	48	45	60
Total	4396	3316	3169	3614
Deleted gene	0	1080	1227	782


Target region for deletion
Cm^s Suc^R
Wild type
SC fragment
sacB
cat
SC deletion strain
Cm^R Suc^S
sacB
cat
PO fragment
Cm^S Suc^R
PO deletion strain


Δ018
1stepA
Δ055
2stepA
Δ062
3stepC
Δ019
3stepB
unitS
3stepA
Δ019
4stepA
unitH
4stepC
Δ062
4stepB
Δ062
5stepB
unitF
5stepA
unit05A
5stepC
unitB
6stepA
unitK
6stepB
unitR
6stepC
unitC
7stepC
unitM
7stepB
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8stepA
Δ054
8stepB
unitQ
8stepC
unitA
9stepA
unitK
9stepB
unitQ
9stepC
unitH＋Δ053
10stepA+
unitK+Δ053
10stepB+
unitQ+Δ053
10stepC+
unitQ
10stepC
unitK
11stepA
unitH
11stepC
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11stepB
unitE
11stepD
unitH
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12stepC
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Δ049
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unitI
14stepB
unitE
14stepD
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Δ098
15setpE
Δ072
15stepA
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15stepF
Δ098
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unitN
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Δ072
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unitT
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unitP
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unitE
17stepA
unitE
17stepC
Δ084
18stepB γ
unitJ
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Δ084
18stepB
Δ091
18stepC
Δ069
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unitD
19stepH
unit19B
19stepA
unit18C
19stepB
Δ069
19stepC
Δ036
19stepC α
Δ043
19stepC β
unitD
19stepD
Δ091
19stepE
Δ012
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unitC
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unitC
20stepD
Δ012
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unitD
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unitD
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Δ017C
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25stepA
Δ045
25stepB
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Δ100
26stepC
Δ100
27stepA
Δ045
27stepB
Δ019
27stepC
Δ019
28stepA
Δ101B
28stepB
Δ062
28stepC
Δ017C
28stepD
Δ103
MGF-01