Supplementary Figure 1: (A) Products after 35 PCR cycles using vector-specific primers that flank the library insertion site. 1 μL of PCR reaction was loaded directly onto a 1.0% agarose gel. Template for lanes 2-5 was 5 ng of *E. coli* pLib1, lanes 7-10 was 5 ng of pIMP1 vector DNA, and lanes 12-15 was 100 ng of *C. acetobutylicum* genomic DNA. Final Mg^{+2} concentrations were 2 mM (lanes 2,7,and 12), 4 mM (lanes 3,8, and 13), 6 mM (lanes 4,9, and 14), and 8 mM (lanes 5,10, and 15). Lanes 1 and 16 contain the λBsteII standard, and lanes 6 and 11 contain the ϕX174-HaeIII standard. (B) Impact of increasing PCR cycles on product mass using 5 ng of *E. coli* pLib1 template. PCR samples were Qiagen purified, eluted in 80 μL 10 mM Tris-HCl, and A_{260} readings taken. (C) Characterization of potential bias due to PCR amplification of library inserts. Amplified library inserts after 17, 19, 21, and 23 PCR cycles were Qiagen purified, labeled with Cy3, and hybridized to DNA microarrays against sonicated, Cy5-labeled, *E. coli* pLib1 DNA.

Supplementary Figure 2: Cell density (A_{600}) and metabolite profiles of *C. acetobutylicum* (pLib1) cultures challenged with 0% (—◆—), 0.62% (—■—), 1.24% (—▲—), or 1.56% (—✕—) 1-butanol. (A) Cell Density. (B) Glucose. (C) Acetate. (D) Butyrate. (E) Net butanol production, calculated by subtracting the initial butanol challenge concentration from each subsequently measured butanol concentration.

Supplementary Figure 3: Signal intensity ranks over the course of butanol challenge transfers for the 10 genes with the highest signal intensity rank as of the 13th transfer for (A) biological replicate experiment 1 and (B) biological replicate experiment 2. Bar color-coding is as follows: □ - Inoculum; ■ - Transfer 4; □ - Transfer 7; □ - Transfer 10; ■ - Transfer 13

Supplemetary Figure 4: Static flask cultures of 824(pCAC1869) (—■—) and 824(pIMP1) (- · - ○ · - ·) in the absence of external butanol stress. (A) A_{600}. (B) Residual glucose. (C) Butanol production.

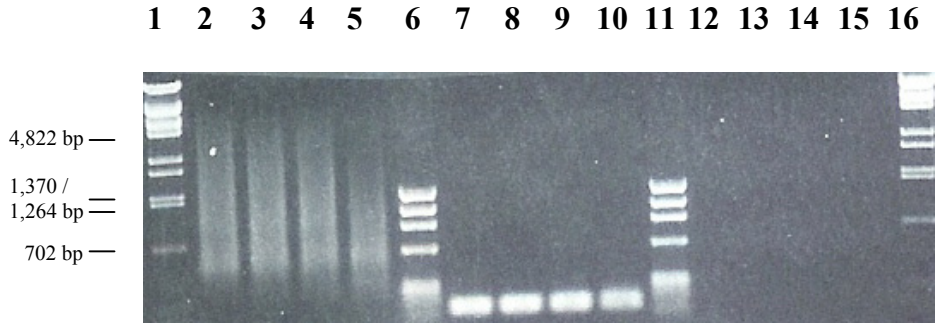
Supplementary Table 1: Unique library inserts identified by sequencing plasmids from individual colonies plated from the 1.56% butanol challenge bottle. Shown are the chromosomal (or megaplasmid) start and end positions of each sequenced insert, the insert length, the identity of the gene in which each insert starts/ends, and whether the start/end positions are inside of an ORF (open reading frame) or an IR (intergenic region).

Supplementary Table 2: Key parameters of stationary-phase transfer challenge experiments (Protocol II). See text for details of experimental design. Butanol challenge concentration, glucose consumption, and metabolite production at each challenge level determined by HPLC are shown. For comparison, the parameters resulting from single-transfer experiments at discrete challenge levels (Protocol I) are also shown. 10 mL test tubes of CGM containing 100 g/L glucose and 100 μg/mL Em were inoculated from each challenge transfer to determine the capacity of stress-selected cultures to produce butanol. These tube cultures were incubated and sampled after 168 hours and again after 216 hours to determine final metabolite levels.

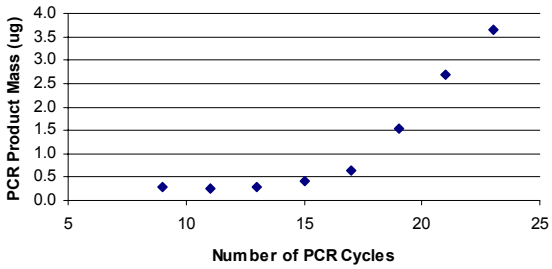
Supplementary Table 3: Plasmid DNA isolated from the challenge inoculum was PCR amplified and hybridized against oppositely labeled, amplified plasmid DNA from the 4^{th}, 7^{th}, 10^{th}, and 13^{th} challenge transfers. Intensities for each gene with signal-noise ratio > 3 were then ranked for each microarray, and a table of genes generated showing the rank of every gene at a given transfer, as well as the average signal intensity of three DNA microarray target-spots per gene.

Supplementary Figure 1

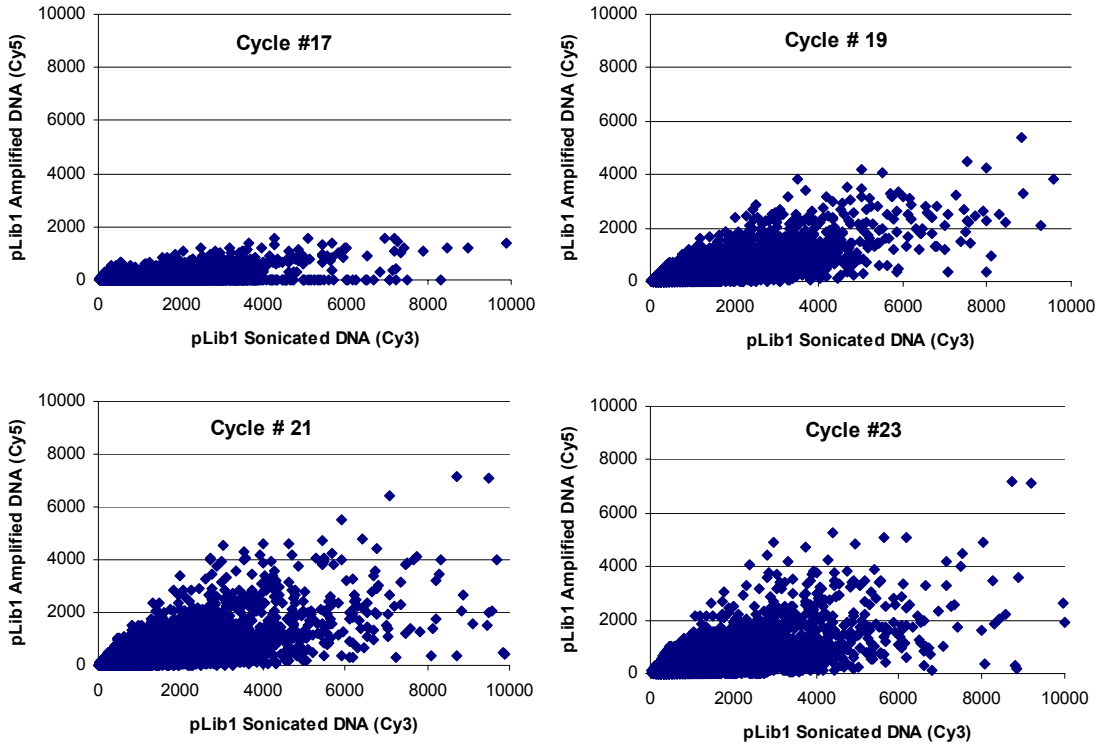
(A)



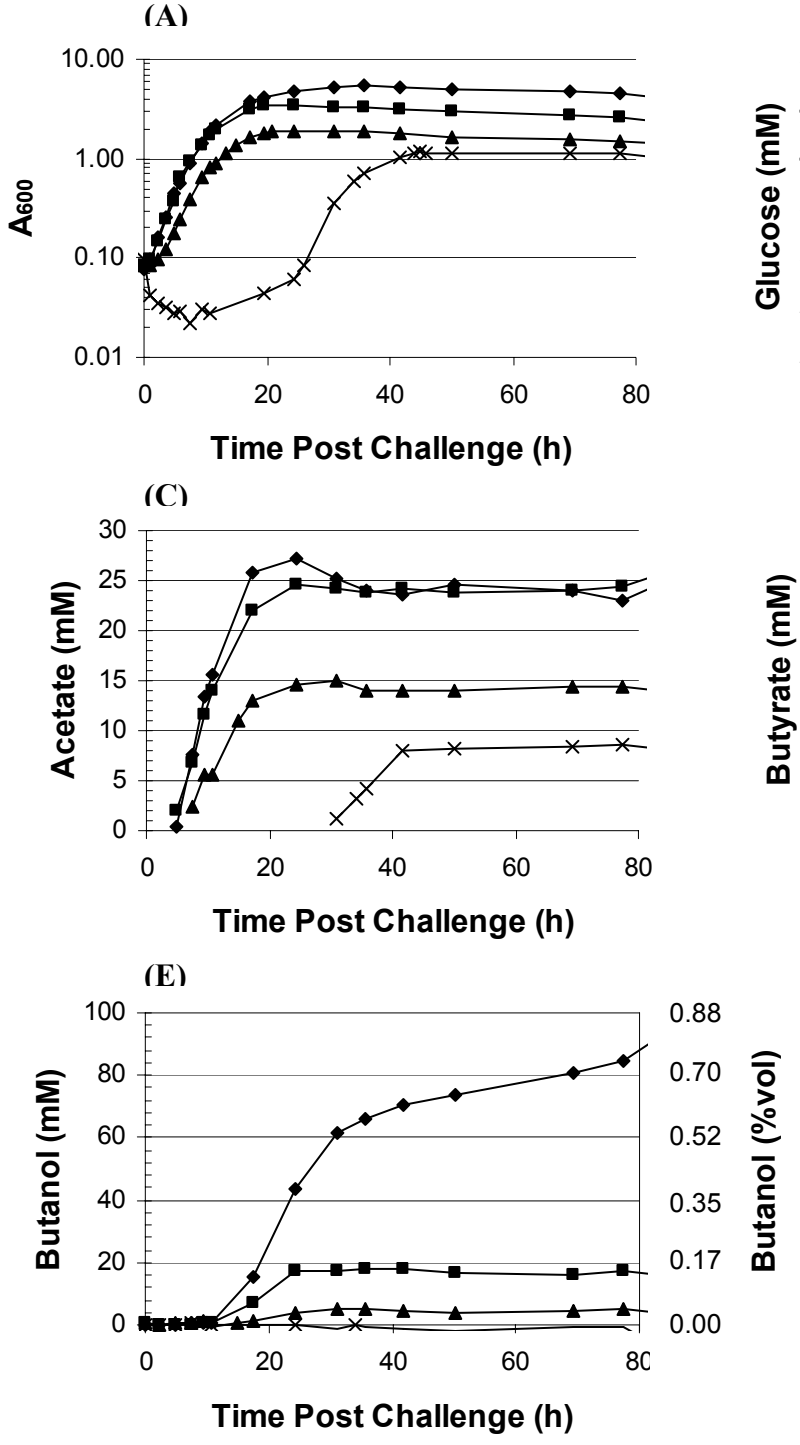
(B)



(C)

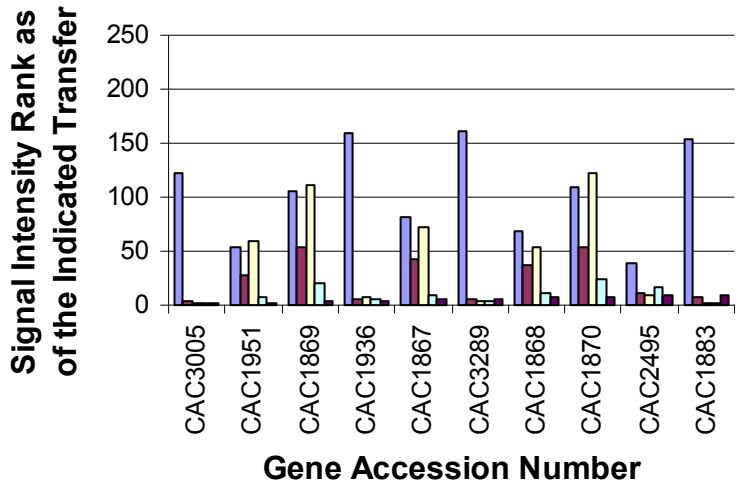


Supplementary Figure 2

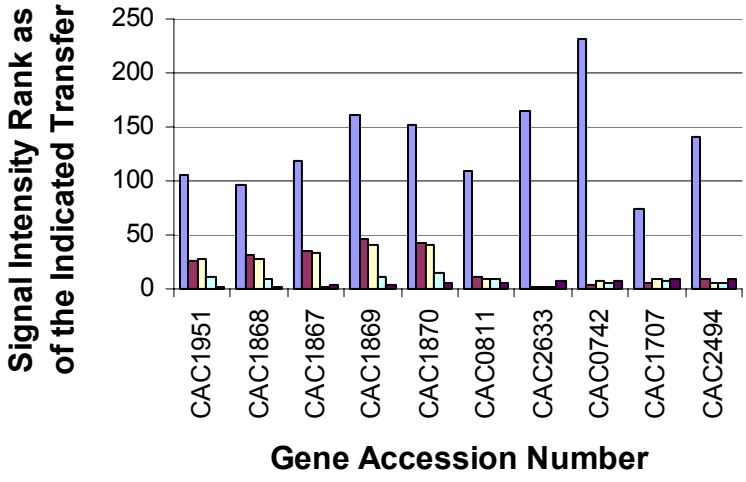


Supplementary Figure 3

(A)

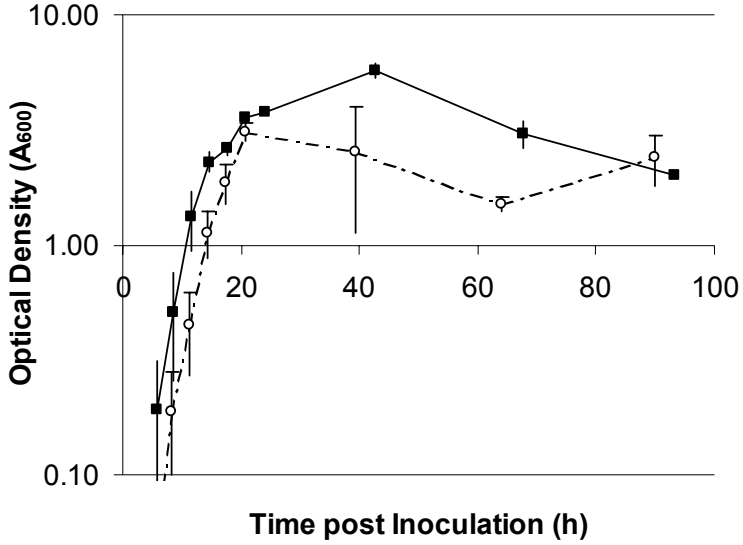


(B)

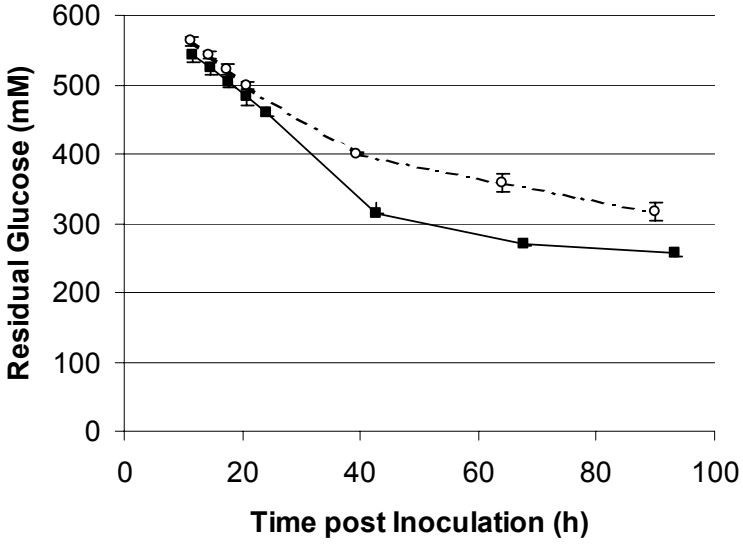


Supplementary Figure 4

(A)



(B)



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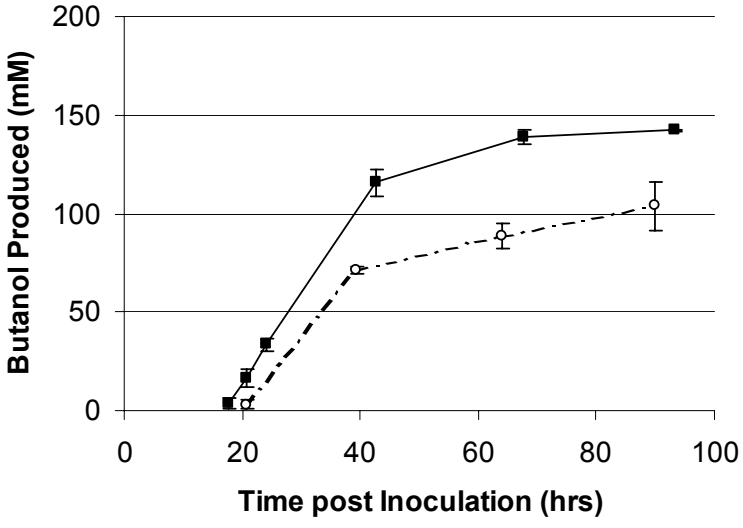

Supplementary Table 1

Insert	Start	End	Length	Insert Start	Start Loc	Insert End	End Loc	# of Clones	Entire ORF?
1	2,991	3,639	648	CAC0002	ORF	CAC0004	ORF	6	Y
2	200,039	200,793	754	CAC0176	ORF	CAC0176	ORF	1	N
3	295,498	295,659	161	CAC0263	ORF	CAC0263	ORF	1	N
4	805,577	806,788	1211	CAC0696	ORF	CAC0697	ORF	4	N
5	1,148,483	1,149,352	869	CAC1002	ORF	CAC1002	ORF	1	N
6	1,267,666	1,268,659	993	CAC1098	ORF	CAC1098	ORF	1	N
7	2,485,906	2,486,445	539	CAC2376	ORF	CAC2377	ORF	2	N
8	2,979,258	2,979,601	343	CAC2846	ORF	CAC2846	ORF	1	N
9	3,056,382	3,057,183	801	CAC2922	ORF	CAC2923	ORF	1	N
10	3,073,532	3,073,776	244	CAC2941	IR	CAC2942	ORF	1	N
11	3,187,582	3,188,104	522	CAC3038	ORF	CAC3038	IR	1	N
12	3,925,011	3,925,086	75	CAC3718	ORF	CAC3718	ORF	1	N
13	49,111	49,252	141	CAP0049	IR	CAP0049	IR	1	N
14	83,161	83,391	230	CAP0073	IR	CAP0073	IR	1	N
								23	

Supplementary Table 2

	Volume % Butanol		mM Glucose Consumed		Final Metabolite Levels						Outgrowth Test Tube Butanol Production	
					Acetate		Butyrate		Butanol*			
Transfer	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1**	Rep2**
1	0.47%	0.47%	248.2	224.8	9.9	9.7	22.3	23.0	111.8	107.7	1.49%	1.51%
3	0.57%	0.55%	201.6	203.8	7.4	7.9	17.4	18.2	63.6	68.0	1.36%	1.48%
4	0.86%	0.74%	176.9	179.9	8.6	8.6	18.1	17.8	59.2	79.8	1.31%	1.51%
9	1.19%	1.25%	98.8	84.6	5.4	7.4	14.0	18.1	28.9	27.9	1.41%	1.57%
10 [1]	1.30%	1.30%	31.0	62.5	9.2	11.5	0.0	21.8	0.0	19.0	1.70%	1.17%
11	1.39%	1.43%	45.8	44.1	10.0	9.9	23.5	26.3	0.0	1.1	1.51%	0.42%
12	1.52%	1.51%	39.7	47.6	6.4	9.2	20.6	25.3	0.0	0.0	1.07%	0.32%
13	1.56%	1.56%	36.7	22.6	8.4	8.2	22.0	20.8	0.0	1.0	1.55%	1.47%
14	1.62%	1.60%	23.2	24.1	3.1	6.8	18.6	19.4	0.0	0.0	1.86%	1.89%
Protocol I Challenges:												
0.00%	-	-	190.0	-	24.7	-	17.3	-	92.3	-		
0.62%	0.62%	-	63.6	-	25.6	-	27.4	-	16.3	-		
1.24%	1.24%	-	30.0	-	14.0	-	20.1	-	3.7	-		
1.56%	1.56%	-	16.8	-	8.2	-	16.0	-	0.0	-		

Rep1 and Rep2 refer to biological replicate challenge experiments 1 & 2

* Butanol value shown is the final measured level minus the initial challenge level

** Butanol production is the average of two-10 mL test tubes of CGM containing 100 g/L glucose and 100 μg/mL erythromycin and inoculated with 100 μL of the the indicated challenge bottle and grown 168-216 h

1 824(pIMP1) plasmid control cultures did not grow in 10th transfer, containing 1.30% butanol

Supplementary Table 3

(A)

Gene	Transfer 13 Rank	Transfer 10 Rank	Transfer 7 Rank	Transfer 4 Rank	Unchallenged Inoculum Rank	Transfer 13 Intensity	Transfer 10 Intensity	Transfer 7 Intensity	Transfer 4 Intensity	Unchallenged Inoculum Intensity
CAC3005	1	1	1	3	123	65059	65054	65075	64994	1210
CAC1951	2	8	60	27	54	65058	51916	1463	11279	5298
CAC1869	3	20	111	53	105	65054	20534	547	3415	1578
CAC1936	4	6	7	6	160	65051	62504	58549	64971	639
CAC1867	5	10	73	43	81	65046	42143	1044	5538	2711
CAC3289	6	3	3	5	161	65045	65025	65052	64974	638
CAC1868	7	11	53	37	68	64944	38488	1707	7385	3663
CAC1870	8	25	123	54	109	64630	17778	461	3104	1532
CAC2495	9	16	9	11	38	62872	23821	53650	47415	8223
CAC1883	10	2	2	7	153	61387	65039	65066	64967	725
CAC2494	11	23	13	19	63	46764	18947	29854	20649	4336
CAC0395	12	5	4	2	173	45310	64514	64202	65007	512
CAC3446	13	4	5	9	214	42498	64984	64113	61588	341
CAC1874	14	7	6	8	151	36018	62112	60991	62918	741
CAC1883	15	9	11	10	167	35693	44684	30635	56128	578
CAC2413	16	12	8	4	130	31406	36267	55975	64979	970
CAC3359	17	18	14	1	268	26942	21288	26277	65009	234
CAC1291	18	19	18	12	166	23177	20854	16776	42389	587
CAC3445	19	14	12	13	308	20246	33500	30566	40939	185
CAC1124	20	34	25	18	158	17106	8568	8413	22792	653
~~CAC0706~~	~~21~~	~~24~~	~~17~~	~~17~~	44	~~15652~~	~~18658~~	~~17580~~	~~23619~~	~~7356~~
CAC1137	22	21	19	21	9	13405	20469	16731	20272	22821
CAC2840	23	22	15	16	336	12777	20180	18257	25342	158
CAC1767	24	26	20	39	697	12318	17648	11332	6839	34
CAC0494	25	27	22	22	100	11705	15802	10106	17229	1694
CAP0139	26	42	54	28	61	9972	5457	1703	10328	4507
CAC1225	27	17	16	24	199	9223	21734	18064	15346	385
CAC3288	28	15	28	32	692	8628	27984	6306	7727	34
CAC1675	29	13	10	14	98	8504	34335	36207	36724	1798
CAC1137	30	31	33	65	30	7253	12009	4048	2482	10195
CAC1935	31	57	44	76	721	6947	3183	2090	1670	25
CAC3184	32	29	24	34	62	6797	13324	9451	7555	4387
CAC0811	33	32	31	58	28	6596	11891	4819	2918	10579
CAC1711	34	33	26	48	41	6477	11093	8265	4588	7918
CAC0865	35	128	64	26	176	5686	717	1362	13700	502
CAC0831	36	40	48	60	56	4939	6306	1846	2819	5233
CAC1976	37	35	62	107	76	4277	7907	1385	931	3099
CAC2851	38	38	29	42	50	4169	6841	5156	6259	6163
CAC0260	39	46	32	49	59	3963	4457	4614	3942	4659
CAC1326	40	44	35	40	45	3938	5077	3696	6783	7255
CAC3309	41	259	#N/A	229	718	3827	210	#N/A	256	26
CAC3358	42	39	30	23	460	3703	6828	5025	16187	82
CAC3360	43	28	38	33	11	3681	14565	3239	7724	21157
CAC2920	44	52	37	66	93	2963	3630	3335	2455	1929
CAC1231	45	49	51	87	113	2824	4023	1741	1335	1403
CAC2342	46	43	55	137	67	2752	5371	1676	632	3688
CAC0892	47	47	101	128	91	2660	4425	684	661	2038
CAC2882	48	55	112	418	242	2525	3404	533	114	273
CAC1692	49	30	21	25	297	2472	13169	10376	14918	197
CAC3112	50	41	70	74	29	2366	5668	1124	1880	10535
CAC1024	51	45	49	94	77	2312	4640	1841	1153	2954
CAC0075	52	61	43	77	39	2301	2834	2108	1668	8148
CAC3525	53	50	34	36	529	2270	3976	3963	7419	64
CAC1931	54	58	50	112	60	2229	3127	1779	842	4534
CAC0740	55	48	36	30	55	2216	4251	3466	8052	5291
CAC3006	56	51	39	56	82	2054	3860	2884	2965	2568
CAC1045	57	63	46	79	86	1948	2633	1992	1637	2262
CAC2056	58	198	#N/A	#N/A	#N/A	1740	326	#N/A	#N/A	#N/A
CAC3526	59	67	40	45	600	1706	2345	2687	5369	51
CAC1726	60	72	58	61	74	1693	1842	1483	2640	3241
CAC2343	61	78	89	118	95	1653	1642	791	798	1912
CAC1417	62	66	67	113	78	1631	2358	1188	838	2843
CAC2917	63	37	56	47	3	1614	6940	1626	4667	38192
CAC3079	64	59	47	59	69	1571	2918	1953	2895	3483
CAC0412	65	64	52	72	12	1551	2468	1732	1985	20044
CAC0449	66	68	61	71	15	1514	2320	1431	1999	17860
CAC0771	67	76	80	105	24	1513	1699	931	965	11229
CAC3379	68	56	42	64	73	1479	3356	2258	2549	3263
CAC2359	69	71	69	83	717	1375	1914	1146	1506	27
CAC3087	70	65	45	63	684	1356	2424	2079	2554	35

Supplementary Table 3

(B)

Name	Transfer 13 Rank	Transfer 10 Rank	Transfer 7 Rank	Transfer 4 Rank	Unchallenged Inoculum Rank	Transfer 13 Intensity	Transfer 10 Intensity	Transfer 7 Intensity	Transfer 4 Intensity	Unchallenged Inoculum Intensity
CAC1951	1	11	28	26	104	65022	31643	2634	2010	506
CAC1868	2	11	53	37	68	65011	38488	1707	7385	3663
CAC1867	3	10	73	43	81	65007	42143	1044	5538	2711
CAC1869	4	20	111	53	105	57217	20534	547	3415	1578
CAC1870	5	25	123	54	109	44286	17778	461	3104	1532
CAC0811	6	32	31	58	28	14780	11891	4819	2918	10579
CAC2633	7	94	100	81	1	13840	1173	686	1582	54835
CAC0742	8	172	164	165	34	9927	434	316	445	8887
CAC1707	9	153	155	85	17	6345	528	337	1359	14618
CAC2494	10	23	13	19	63	5795	18947	29854	20649	4336
CAC3134	11	79	86	101	18	5286	1355	862	1021	13075
CAC0767	12	89	92	131	7	4058	1205	762	644	25008
~~CAC0706~~	~~13~~	~~24~~	~~17~~	~~17~~	44	~~3396~~	~~18658~~	~~17580~~	~~23619~~	~~7356~~
CAC0854	14	132	119	136	22	3178	689	478	634	11790
CAC1463	15	274	209	285	237	2389	190	202	178	292
CAC2495	16	16	9	11	38	2373	23821	53650	47415	8223
CAC0977	17	116	96	98	40	2283	854	718	1057	8016
CAC3435	18	82	65	92	43	2008	1325	1345	1197	7433
CAC2409	19	186	238	288	49	1632	368	174	177	6764
CAC2294	20	#N/A	#N/A	#N/A	#N/A	1261	#N/A	#N/A	#N/A	#N/A
CAC1146	21	415	332	369	195	1194	102	113	130	407
CAC3310	22	97	90	99	33	1160	1133	773	1035	8963
CAC3485	23	126	202	57	178	902	743	213	2926	490
CAC3360	24	28	38	33	11	883	14565	3239	7724	21157
CAC2727	25	#N/A	#N/A	#N/A	#N/A	833	#N/A	#N/A	#N/A	#N/A
CAC1711	26	33	26	48	41	820	11093	8265	4588	7918
CAC0432	27	60	131	90	124	798	2890	432	1220	1152
CAC0831	28	40	48	60	56	710	6306	1846	2819	5233
CAC2754	29	289	325	279	119	692	172	116	183	1267
CAC2056	30	198	#N/A	#N/A	#N/A	648	326	#N/A	#N/A	#N/A
CAC3485	31	202	166	52	162	608	308	315	3533	622
CAC3006	32	51	39	56	82	603	3860	2884	2965	2568
CAC2917	33	37	56	47	3	600	6940	1626	4667	38192
CAC1225	34	17	16	24	199	595	21734	18064	15346	385
CAC0757	35	282	320	473	133	537	177	118	103	958
CAP0050	36	86	82	108	25	521	1251	910	926	11174
CAC1147	37	705	418	559	384	519	65	90	95	123
CAC0200	38	80	97	142	20	512	1336	715	571	12280
CAC0583	39	786	467	355	520	510	55	84	136	66
CAC1093	40	115	129	80	90	468	903	440	1592	2091
CAC0901	41	180	552	395	206	421	407	75	120	370
CAC2667	42	315	193	122	37	421	151	230	744	8463
CAC3317	43	328	#N/A	#N/A	#N/A	419	140	#N/A	#N/A	#N/A
CAC1936	44	6	7	6	160	396	62504	58549	64971	639
CAC0153	45	117	161	214	186	395	846	324	276	460
CAC0407	46	285	298	371	101	376	176	125	129	1691
CAC0892	47	47	101	128	91	302	4425	684	661	2038
CAC3005	48	1	1	3	123	293	65054	65075	64994	1210
CAC2343	49	78	89	118	95	272	1642	791	798	1912
CAC1132	50	112	110	95	127	263	930	554	1114	1046
CAC1045	51	63	46	79	86	259	2633	1992	1637	2262
CAC1976	52	35	62	107	76	245	7907	1385	931	3099
CAC2410	53	360	343	270	79	241	125	109	192	2841
CAC3002	54	468	393	611	359	235	87	94	89	137
CAC1863	55	338	329	344	142	221	137	114	142	832
CAC0303	56	113	88	73	70	218	930	821	1981	3455
CAC0564	57	268	218	333	253	214	200	195	148	256
CAC3446	58	4	5	9	214	214	64984	64113	61588	341
CAC1126	59	318	273	67	258	211	150	139	2348	245
CAC1940	60	292	331	268	256	207	169	113	197	250
CAC0031	61	514	435	678	522	206	80	88	83	65
CAC1675	62	13	10	14	98	195	34335	36207	36724	1798
CAC1254	63	299	528	484	180	194	164	77	102	477
CAC0342	64	#N/A	#N/A	#N/A	#N/A	190	#N/A	#N/A	#N/A	#N/A
CAC1988	65	618	613	125	502	188	71	63	680	71
CAC1722	66	388	529	578	519	188	109	77	93	66
CAC2177	67	62	99	68	47	185	2776	709	2341	7167
CAC3669	68	675	383	192	376	184	68	96	322	126
CAC0473	69	525	468	513	134	171	79	84	99	947
CAC0094	70	#N/A	#N/A	#N/A	#N/A	171	#N/A	#N/A	#N/A	#N/A